

**The Role of Inhibins and Activins in
Follicle Development in the Sheep**

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Declaration

The research described in this thesis, except where reference has been made, is solely the work of the author and has not been submitted for any other degree or professional qualification at the University of Edinburgh or any other educational institution.

Alexander James Mitchell

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And now for the science...

Publications Arising from this Thesis

Meeting Abstracts

Mitchell AJ, Campbell BK, Groome NP and Baird DT 2000 Source of inhibin A throughout the ovine oestrous cycle. *Journal of Reproduction and Fertility*, **Abstract Series 25**, abstract number 198.

Mitchell AJ, Campbell BK, Groome NP and Baird DT 2001 Activin subunits diffuse from follicles throughout the sheep ovary. *Reproduction*, **Abstract Series 27**, abstract number 73.

Research Paper

Mitchell AJ, Souza CJH, Campbell BK, Groome NP and Baird DT 2002 Activin subunits may diffuse from follicles throughout the sheep ovary. *Submitted to Reproduction*, May 2002.

Abstract

The growth and development of ovarian follicles is controlled primarily by FSH and LH secreted by the anterior pituitary. In the ewe, the secretion of FSH and LH by the pituitary is regulated by a number of ovarian factors, including oestradiol, progesterone, and the glycoprotein dimers inhibin and activin. Two forms of inhibin have been identified to date, inhibin A and inhibin B, with both consisting of a common α -subunit and differing β -subunits. Although well researched in a number of species, the levels of inhibins in the circulation and the expression of their subunits in the gonads have yet to be examined as thoroughly in the sheep, a species in which the ovulatory quota is rigorously controlled.

In order to identify the source of inhibin A in the ewe, ovaries were removed at each of three points during the oestrous cycle (mid-luteal, early follicular and late follicular), all visible antral follicles dissected out and hormone release measured during incubation and in their follicular fluids. Large oestrogenic follicles were found to be the main source of inhibin A in the ewe with large non-oestrogenic follicles also releasing significant amounts of the hormone. The levels of inhibin A released by both small antral follicles and large oestrogenic follicles during the early follicular phase were significantly reduced compared to the luteal phase, possibly reflecting the fall in FSH concentration at this time.

Conventional immunocytochemistry and dual label confocal microscopy were used to investigate the expression of inhibin α , β A and β B subunit proteins in different compartments of the ovarian follicle. All three subunits were found in the granulosa cells of antral follicles with little variation in the relative amounts of the proteins in follicles during the oestrous cycle. In addition, there was significant immunostaining for both β -subunits (but not α subunit) in theca cells, with mRNA expression of all three subunits confined to the granulosa cells. These findings are compatible with the hypothesis that β A and β B activins diffuse from the granulosa layer through the theca and possibly the adjacent stroma, where they may exert a paracrine influence.

Significant amounts of inhibin B have not been detected in the blood or follicular fluid of sheep using a specific two-site ELISA assay. The nature of

inhibin/activin proteins present in ovarian cells and follicular fluid was therefore investigated using Western blotting. A small amount of high molecular weight protein (consistent with inhibin B precursor) was detected in protein extracts of ovarian cells. Conversely, there were no inhibin B proteins present in follicular fluid, which contained large quantities of inhibin A forms. Therefore, although all three inhibin subunit mRNAs and proteins are expressed in the granulosa cells of antral follicles, it seems unlikely that inhibin B is secreted outside the cell.

In order to explore further the forms of inhibin produced by the sheep, subunit expression in the male was investigated. In most species studied (e.g. human, rat), the male secretes exclusively inhibin B. In this work, both α and β B subunit proteins were found in the seminiferous tubules of rat testes. In contrast, all three subunit proteins were present in the testis of the ram, which like the ewe, apparently only secretes inhibin A. Together, these findings are consistent with the hypothesis that β A subunit is preferentially bound to α subunit when both β subunits are expressed in the species studied, and that inhibin B is only secreted in the absence of β A protein.

To further examine the hypothesis that inhibin and activin may influence ovarian function through paracrine and autocrine actions, differences in the expression of inhibin subunits in ewes carrying the fecundity gene, which leads to precocious follicular maturation, were examined. It was noted that expression of both α and β A subunits was significantly lower in medium sized antral follicles in carriers of the mutation. Furthermore, the level of diffusion of β B subunit from medium and large antral follicles was significantly higher in animals carrying the fecundity gene. The level of β B-subunit associated proteins diffusing from the granulosa cells is increased in antral follicles, possibly reflecting an increase in activin diffusion which may affect the growth of smaller follicles.

In conclusion, this work has 1) identified the sources of inhibin A in the ovine ovary; 2) provided evidence for the possible diffusion of activins throughout ovarian tissue; 3) supported the hypothesis that the ewe does not produce inhibin B; 4) reported a possible increase in activin diffusion from specific follicle populations in ewes with increased ovulation rates; and finally 5) shown that in some species there may be preferential binding of certain inhibin subunits during dimerisation.

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List of Abbreviations

A ₄	Androstenedione
ALK	Activin-like Kinase
AMH	Anti Müllerian Hormone
BMP	Bone Morphogenetic Protein
BMPR	BMP Receptor
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary DNA
CL	<i>Corpus Luteum</i>
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
E ₂	Oestradiol
ECF	Enhanced Chemifluorescence
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked immunosorbent assay
FF	Follicular Fluid
FITC	Fluorescein Isothiocyanate
FSH	Follicle Stimulating Hormone
FS/FSP	Follistatin
GDF	Growth/Differentiation Factor
GnRH	Gonadotrophin-Releasing Hormone
HFF	Human Follicular Fluid
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
INF- γ	Interferon- γ
InhBP	Inhibin Binding Protein
LH	Luteinising Hormone
mRNA	Messenger RNA
NSB	Non-specific Binding

OE	Oestrogenic
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PVDF	Polyvinylidene Fluoride
QC	Quality Control
RNA	Ribonucleic Acid
s.c.	Subcutaneous
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
SSC	Sodium Chloride/Sodium Citrate Solution
StAR	Steroid Acute Regulatory protein
TBS-T	Tris-Buffered Saline/Tween Solution
TC	Total Counts
TE	Theca Externa
TGF β	Transforming Growth Factor – β
TI	Theca Interna
TRITC	Tetramethyl Rhodamine Isothiocyanate

Chapter 1 - Literature Review

Folliculogenesis, the process of follicle development to a pre-ovulatory stage, is known to be regulated by a large number of endocrine, paracrine and autocrine factors. The growth of follicles is initially reliant upon local ovarian stimuli with dependency shifting to the gonadotrophins FSH and LH later in development.

Selection of a particular follicle from a numerous cohort is required for ovulation to occur during the oestrous cycle. This selection involves intricate signalling via ovarian molecules and pituitary gonadotrophins, resulting in the development of a single ovulatory follicle in monoovular species with polyovular animals maintaining several preovulatory follicles.

Although the roles of a majority of the molecules involved in this process have been extensively researched, at present the precise mechanisms surrounding the phenomenon of follicle selection have yet to be elucidated.

For the purpose of this review, the sheep will be the main species discussed with comparative references to a number of other animals.

1.1 Folliculogenesis

In the sheep, adult ovaries contain a follicular reserve of between 12,000-86,000 primordial follicles (at two years of age) with a further 50-400 follicles at various stages of development (Turnbull *et al.*, 1977) (Cahill *et al.*, 1979).

The formation of primordial follicles from germ cells takes place during fetal life after the migration of germ cells to the genital ridge and differentiation of the gonads has taken place (Mauleon, 1978). Members of the TGF β protein superfamily are reported to be essential in the early development and organization of the ovary, with bone morphogenetic proteins 4 and 8 (BMP4/8), produced by neighbouring somatic cells, being key factors in the generation of the primordial germ cells (Ying *et al.*, 2000).

Oocytes, which are arrested in prophase I of meiosis, are enclosed in a layer of flattened pre-granulosa cells leading to the formation of the first primordial follicle around 75 days into gestation (Peters *et al.*, 1975) with 91% of the oocytes

incorporated into primordial follicles by day 135, just prior to birth. This is in contrast to early follicular development in other species such as rodents and rabbits, where the primordial pool develops in the early neonatal period (Marion and Gier, 1971).

The oocyte itself plays a highly significant role in the formation of a primordial follicle. The recent discovery of the transcription factor Fig α (factor in the germline α) has identified an oocyte specific molecule necessary for the formation of a primordial follicle (Soyal *et al.*, 2000). Fig α induces the expression of proteins which contribute to the zona pellucida (ZP), and it has been suggested (Richards, 2001) that the expression of the ZP proteins may be essential for the physiological connection of the oocyte to pre-granulosa cells.

When the primordial cohort has developed, follicles are gradually selected for further growth and small numbers constantly leave the quiescent stage and resume growth.

In the ovine ovary, it is estimated that eight primordial follicles disappear from the follicular reserve every day, and that only two or three of these follicles continue their growth, with the other five or six being lost through cell death (Driancourt *et al.*, 1985). The length of time taken for an ovine recruited follicle (with three layers of granulosa cells) to reach an ovulatory stage was originally estimated to be approximately 6 months (Cahill and Mauleon, 1980), however, recent studies using an autograft model have shown that this period may be as short as 2-3 months (Campbell *et al.*, 2000).

McGee and Hsueh (McGee and Hsueh, 2000) suggest that the process of folliculogenesis can be regarded as a continuous activation of resting follicles from the quiescent reserve, termed initial recruitment, with waves of hormone stimulation leading to further cyclical recruitment of antral follicles throughout the oestrous cycle.

1.1.1 Initial recruitment

When a primordial follicle is activated, RNA synthesis occurs along with the proliferation of the pre-granulosa cells surrounding the oocyte (Picton, 2001). The follicle is now committed to further growth and cannot regress to its prior quiescent

state. These activated follicles are now destined to either ovulate or become atretic, the fate of the vast majority being the latter (McNatty *et al.*, 1982).

Relatively little is known about the mechanism of initial follicle recruitment, however, in many mammals there is a distinct relationship between the number of follicles in the quiescent state and the number of growing follicles (Gougeon *et al.*, 1994). In the ovaries of young mammals, the follicular reserve is large and thus the number of growing follicles is high. As the ovary ages the number of growing follicles declines along with the size of the reserve. Conversely, the proportion of early growing follicles in murine and primate ovaries increases with age (Krarup *et al.*, 1969; Gougeon *et al.*, 1992), suggesting the possible removal of an autocrine inhibitory factor produced by resting primordial follicles. This theory is supported by research showing that growth is initiated in a high percentage of bovine primordial follicles *in vitro*, compared to that seen *in vivo* (Wandji *et al.*, 1996), suggesting that factors originating from developing follicles are responsible for the inhibition of recruitment.

The factors involved in this gradual and seemingly tightly controlled process remain largely unknown and along with the suggestion of a decline in an inhibitory factor, numerous stimulatory factors have been identified. The origin of these molecules seems to be the ovary itself, as recruitment to growth is possible in the absence of pituitary-derived hormones in hypophysectomised animals (McNatty, 1992). Recent research has also indicated that vascularizing pieces of ovarian cortex *in vivo* maintains follicular quiescence, whereas *in vitro* culture allows unrestrained activation of primordial follicles (Fortune *et al.*, 2000). There are numerous putative factors which have been put forward as candidate recruitment initiators, including basic fibroblast growth factor (van Wezel *et al.*, 1995), nerve growth factor (Dissen *et al.*, 2001), c-kit and its ligand (Yoshida *et al.*, 1997), and activin-A together with components of the extracellular matrix (Oktay *et al.*, 2000). In contrast, there have been other molecules identified, such as anti-Mullerian hormone, which inhibit the initiation of growth in primordial follicles (Durlinger *et al.*, 1999). Growth/differentiation factor-9 (GDF-9) is a growth factor expressed exclusively by the oocyte and is present at all stages of follicular development, except in primordial follicles (McGrath *et al.*, 1995). Studies examining GDF-9 deficient mice recorded

normal primordial and early primary follicle growth, however, the knockout caused a total block in development beyond this stage, leading to complete infertility (Dong *et al.*, 1996). Oocyte growth and the formation of the zona pellucida were unaffected in these animals but significant changes in oocyte differentiation were noted.

Hirshfield (1991) suggested that recruited follicles are able to grow and develop readily in the presence of basal levels of gonadotrophins, metabolic hormones and growth factors until a certain point. They then reach the end of their lifespan under these conditions and for continued growth to occur, and for follicles to avoid becoming atretic, they must receive additional specific signals.

1.1.2 Cyclic recruitment (Selection)

Once recruited, early follicles are able to grow due to trophic stimuli from local ovarian factors (review; Roche, 1996). These follicles (0.1 to 2.5mm in the sheep) are late preantral and small antral follicles, which express FSH receptors on their granulosa cells and LH receptors on their theca cells, and are able to generate a trophic response in the presence of gonadotrophins (McNatty, 1992). This class of follicles is thus termed gonadotrophin-responsive, as gonadotrophins are not essential for their growth and development (Cahill, 1981). However, as follicle growth continues the small antral follicles become increasingly reliant upon FSH and LH as their trophic stimulants.

Shortly after the formation of the antrum, filled with follicular fluid, which occurs when the follicle is between 0.2-0.4mm (Turnbull, *et al.*, 1977), the follicular cells begin to grow and differentiate rapidly. This considerable increase in growth when the follicle is between 0.7 and 1.5mm in diameter is highlighted by a high mitotic index at this time, which reaches a peak at 0.85mm (Cahill and Mauleon, 1980) and then decreases gradually until reaching near zero in preovulatory follicles.

Research has shown that follicles with a diameter greater than 2.5 mm require gonadotrophins for continued growth (Dufour *et al.*, 1979) and that FSH is more essential during this period than LH. Although FSH is the more important gonadotrophin in early follicular development, with LH becoming increasingly significant later in the process, both hormones exert their effects in follicular cells via the cyclic-AMP (cAMP) signalling pathway (Yong *et al.*, 1992). This dual-signalling

through the same molecule is possible due to the variation in cAMP tone within the cell. A low tone, associated with FSH, seems to favour aromatase expression and cell growth, with LH inducing a high cAMP tone, favouring progesterone production and a decrease in cell growth.

In addition to gonadotrophins, the insulin-like growth factor (IGF) system has been shown to play a key role in the regulation of follicle development at the point follicles become responsive to FSH and LH. IGF-1 has been shown to potentiate the action of FSH on granulosa cells by increasing the expression of FSH receptor in murine ovaries (Zhou *et al.*, 1997). The importance of the IGF system in follicular survival is highlighted by the fact that healthy follicles have a high level of bioavailable IGF in their follicular fluids, in contrast to atretic follicles which have an increased level of IGF-binding proteins (IGFBPs) and therefore reduced levels of free IGF (Monget *et al.*, 1996). In addition, large oestrogenic follicles have very low levels of IGFBP-2 (Armstrong *et al.*, 1998).

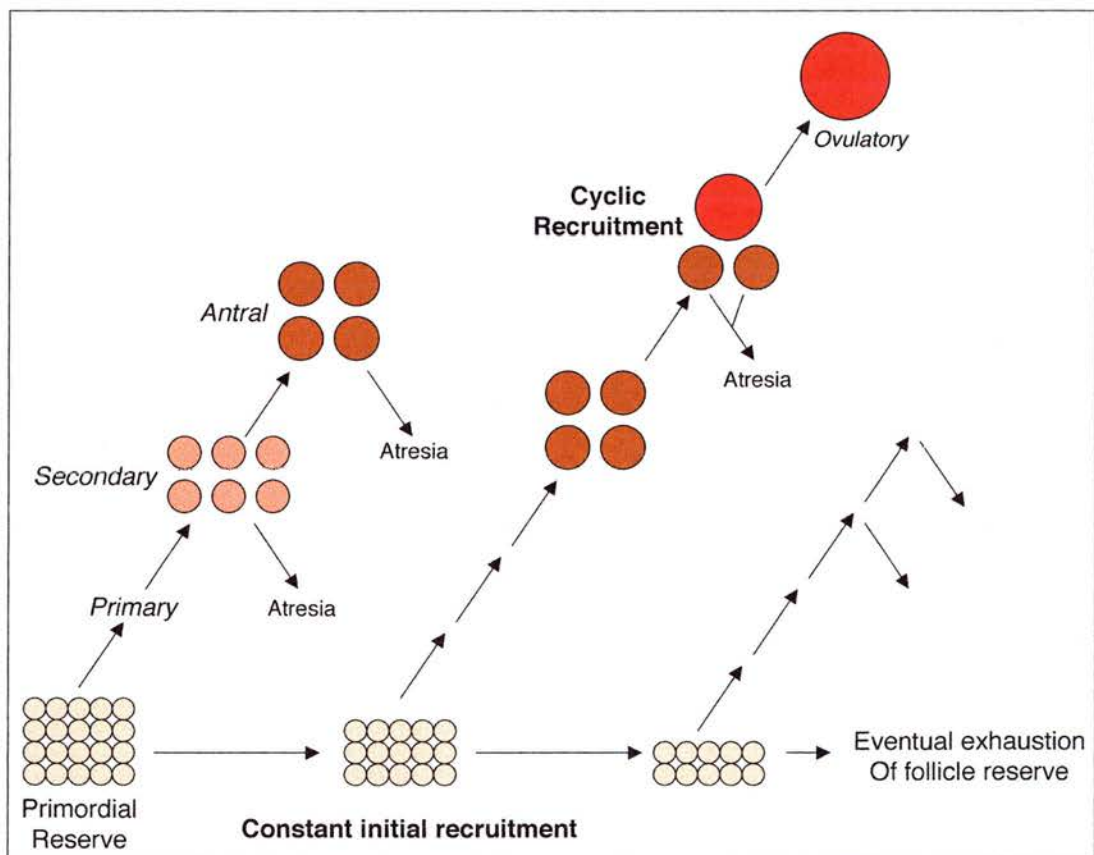


Figure 1.1 Initial and cyclic recruitment of follicles within the ovary, showing the continuing depletion of primordial follicles and regular selection of ovulatory follicles from a secondary cohort. (Modified from McGee and Hsueh, 2000).

1.1.3 Dominance

Early research in the sheep (Smeaton and Robertson, 1971) identified the development of a follicle of preovulatory size at three points during the cycle; day 6-9, day 13 and 1-2 days before the onset of next oestrus. The growth of these follicles is due to waves of cyclical recruitment occurring at regular intervals throughout the oestrous cycle (Evans *et al.*, 2000), leading to the repeated development of a cohort of large antral follicles. In response to the hormonal environment one or more of these follicles are able to become 'dominant' over the other selected antral follicles.

The dominant follicle is so called as increasing evidence suggests that once selected it is able to suppress further growth by other subordinate follicles within the ovine ovary (Campbell *et al.*, 1999a; Driancourt *et al.*, 2000). The expression of LH receptors on the granulosa cells (Webb and England, 1982) and increased oestradiol secretion are markers of the dominant follicle and are of paramount importance to its survival. The increased vascularisation of the theca compartment of the follicle is also likely to play a part in the ability of the follicle to continue growth.

The theca cells of dominant follicles in the cow release greater quantities of androgen, and granulosa cells are able to convert significantly more androgen to oestradiol than the same cells from non-dominant follicles (Turzillo, 1992).

The dominant follicle is able to avoid the atretic fate of the other recruited follicles due to its capacity to survive the low levels of FSH, induced by increasing levels of both ovarian oestradiol and inhibin. The follicle destined to become dominant may be able to generate trophic cAMP levels within the granulosa in response to LH (due to the expression of LH receptors on the granulosa cells) utilising it as a surrogate for declining FSH (Yong, *et al.*, 1992). Research has shown that ovulatory follicles are able to transfer their dependency from FSH to LH in order to survive the switch in gonadotrophic support (Campbell *et al.*, 1999b). The capability of these follicles to make this change is therefore thought to be essential to the mechanism of follicle selection. Additionally, recent research working with bovine follicles has identified the level of insulin-like growth factor binding protein-4 (IGFBP-4) as a predictor of the dominant follicle (Fortune *et al.*, 2001). A decrease in this protein caused by FSH-induced up-regulation of IGFBP-4 protease in dominant follicles leads to greater bioavailability of IGF, which in turn enhances the follicles

responsiveness to FSH. The ability of specific follicles to decrease levels of IGFBP-4 is thought to be essential to the emergence of dominant follicles.

The transition of the dominant follicle to a preovulatory follicle, which is capable of initiating a surge of LH from the pituitary, may be due to the increase in LH pulse frequency during the follicular phase. This additional stimulus from LH is not present during the luteal phase and may explain why dominant follicles are unable to produce the levels of oestradiol necessary for the instigation of an LH surge and thus ovulation at this time.

It is clear that the follicular environment is responsible for the selection of certain follicles for continued development and the aversion of atresia, but not why one follicle is preferred to another at the same stage of growth.

1.1.4 Atresia

The fate of the vast majority (>99%) of ovarian follicles is to undergo the process of cell loss known as atresia (Arai, 1920), the underlying mechanism of which is apoptosis (Hughes and Gorospe, 1991). Atresia begins with the ovum undergoing cytolysis and the membrana granulosa is cast off into the follicular fluid and eventually reabsorbed. The theca cells surrounding the follicle proliferate and form a yellowish shell and the follicular fluid begins to dissipate from the antrum. Eventually the follicular walls collapse leaving an entity similar to that found at the latest stages of corpus luteum degradation. There are numerous morphological markers of follicular atresia, including detachment and degeneration of the granulosa cell layer (Hughes and Gorospe, 1991), the appearance of pyknotic nuclei in the granulosa compartment (Hirshfield and Midgley, 1978), fragmentation of the basal lamina (Bagavandoss *et al.*, 1983), decreased follicular oestradiol production (Carson *et al.*, 1981) and decreased binding of FSH and LH (Uilenbroek *et al.*, 1980).

Furthermore, research has shown that messenger RNAs for gonadotrophin receptors and P450 aromatase (Tilly *et al.*, 1992), are selectively decreased in atretic porcine follicles and that apoptosis occurs in both the theca and granulosa cell types. Recent studies have identified the roles of the cell death promoters P53 and Bax (Zwain and Amato, 2001), which are both induced by cyclic AMP (cAMP) in atretic follicles, and the Fas pathway (Porter *et al.*, 2001) in the regulation of follicular

atresia. The level of Fas and Fas-ligand (FasL) are significantly higher in atretic follicles and subordinate follicles have an increased sensitivity to FasL.

1.2 Gonadotrophins

Fevold and colleagues (Fevold, 1931) first reported that a pituitary gonadotropic fraction from postmenopausal women contained two active components, follicle stimulating hormone (FSH) and luteinising hormone (LH).

Secretion of FSH and LH from the anterior pituitary is stimulated by gonadotrophin-releasing hormone (GnRH), a decapeptide secreted by hypothalamic neurons. Release of GnRH has been shown to be influenced by numerous hypothalamic factors including noradrenaline, adrenaline, catecholamines and more recently activin-A (Calogero *et al.*, 1998).

FSH and LH are glycoprotein hormones composed of two subunits, α and β . The α subunit is conserved between the two molecules and is linked non-covalently to one of two structurally unique β subunits which confer a high degree of biochemical and biological specificity to the hormones (Mercer and Chin, 1995). FSH and LH are produced by gonadotrope cells, which account for 7-10% of all cells in the anterior pituitary gland (Marshall, 1989). The physiological half-life of the two hormones differs considerably, with LH measurable in ovine circulation for approximately 20-30 minutes and FSH exerting its effects for 130-160 minutes (McNeilly, 1988). However, if sufficient quantities of gonadotrophin are released by the pituitary or introduced experimentally, the hormones may be biologically active for longer periods.

1.2.1 Gonadotrophin Secretion

The pattern of release of the two gonadotrophins is considerably different. LH is released by the anterior pituitary gonadotropes in a pulsatile fashion through a regulated pathway (Farnworth, 1995) in response to a rise in the level of intracellular calcium caused by the binding of GnRH to the gonadotrope.

FSH, unlike LH is not stored in considerable amounts in the gonadotropes and 60-80% of FSH produced daily is secreted into the circulation, compared with 1-8% of total LH synthesized (Fraser *et al.*, 1981; McNeilly *et al.*, 1982; McNeilly, 1985).

The release of FSH is therefore primarily constitutive with the small amount of protein stored being released along with LH in response to a pulse of GnRH from the hypothalamus.

Release of gonadotrophins from the pituitary is primarily induced by GnRH pulses from the hypothalamus. However, several other factors are able to modulate their secretion. It has been shown in the sheep that FSH release is potentiated by a negative feedback loop involving oestradiol and inhibin (Baird *et al.*, 1991). These two ovarian products act by down-regulating FSH subunit mRNA transcription in the gonadotropes (Carroll *et al.*, 1991a). Oestradiol, together with progesterone has the capacity to act in a negative feedback system to suppress the release of LH from gonadotrope cells (Karsch *et al.*, 1987). In cultured ovine pituitary cells, it has been shown that oestradiol affects a specific number of predetermined cells to inhibit FSH synthesis and increase responsiveness to GnRH (Ghosh *et al.*, 1996). In contrast, inhibin is able to completely abolish FSH production in some cells whilst modulating GnRH and LH receptor expression in others.

1.2.2 Gonadotrophin action

Granulosa cells in the female, and Sertoli cells in the male are the sole target cells for FSH action and are the only cells which express FSH receptor (Simoni *et al.*, 1997). FSH and LH exert their effects on their target cells by binding to specific hormone receptors, both of which are members of the G-protein coupled receptor family (Ulloa-Aguirre and Timossi, 1998). These receptors are glycoproteins characterized by an external N-terminal ligand binding domain, seven α -helical transmembrane domains and several intracellular domains, the last of which is involved in G-protein linked signal transduction and activation of the adenylyl cyclase-protein kinase A signalling pathway (Marsh, 1976; Ratoosh and Richards, 1985; Reichert and Dattatreya Murty, 1989). FSH and LH are also able to increase intracellular calcium ion concentrations, activating calmodulin kinase and protein kinase C signalling pathways respectively (Gudermann *et al.*, 1992; Flores *et al.*, 1998).

Shortly after the ligand binds to its receptor, and intracellular signalling has been initiated, the ligand-receptor complex is internalised by endocytosis and broken

down by lysosomes. The free receptor may then be recycled back to the cell membrane by exocytosis (Catt, 1996).

1.3 Steroidogenesis in the Follicle

Steroidogenic pathways present in the granulosa and theca cells of ovarian follicles are responsible for the synthesis of the systemically active sex-steroids which directly and indirectly influence follicular growth and development.

When an ovine ovarian follicle reaches a diameter of around 3 mm, a dramatic shift in steroid production occurs. Prior to this point, the levels of both oestradiol (E_2) and androstenedione (A_4) are relatively low compared with testosterone (Carson, *et al.*, 1981). The shift in steroid synthesis leads to a rapid increase in oestradiol production and a decrease in the level of testosterone, coinciding exactly with the first expression of LH receptor on the granulosa cells (Carson *et al.*, 1979). However, not all follicles are able to respond in this way and although their steroid content remains comparable with healthy follicles, they do not show the increase in oestradiol output characteristic of healthy follicles (Tsonis *et al.*, 1984a) and undergo atresia after the ovulatory follicle has been selected.

The steroidogenic pathway in follicles is split between the granulosa and theca cell compartments (Figure 1.2), with both cell types being necessary for the production of oestradiol (Hsueh *et al.*, 1984).

Ovarian steroid production relies on both *de novo* production of cholesterol as well as the utilization of cholesterol from the circulation, with the process being controlled by both systemic and locally produced trophic hormones. Acquisition of cholesterol is the first rate-limiting step in the synthesis of ovarian steroids. Delivery of cholesterol into follicular cells is controlled by the hormonally inducible (Clark *et al.*, 1994) steroidogenic acute regulatory protein (StAR). The StAR protein is responsible for the translocation of cholesterol from the cytoplasm of the cell to the inner mitochondrial membranes, where the first steps of steroid synthesis occur. Cholesterol is obtained primarily from lipoproteins in the serum which bind to specific membrane receptors enabling its uptake and storage (Ikonen, 1997).

The first enzymatic step in the production of steroids involves cytochrome P450 cholesterol side-chain cleavage (P450_{sc}) in the conversion of cholesterol to

pregnenolone, in a reaction which is induced primarily by LH and also by insulin-like growth factor I (IGF-1) and FSH (deMoura *et al.*, 1997; LaVoie *et al.*, 1999).

For the synthesis of progesterone to occur, another enzyme, 3 β -hydroxysteroid dehydrogenase (3 β -HSD) needs to be present. Further processing of the steroid is carried out by cytochrome P450 17 α -hydroxylase (P450_{c17}). In ovine, porcine and bovine follicles this enzyme is found exclusively in the theca interna cells (Conley *et al.*, 1995) and is responsible for the generation of the main ovarian androgens, androstenedione and testosterone, by cleaving 21 carbon steroids (progesterone) to 19 carbon androgens.

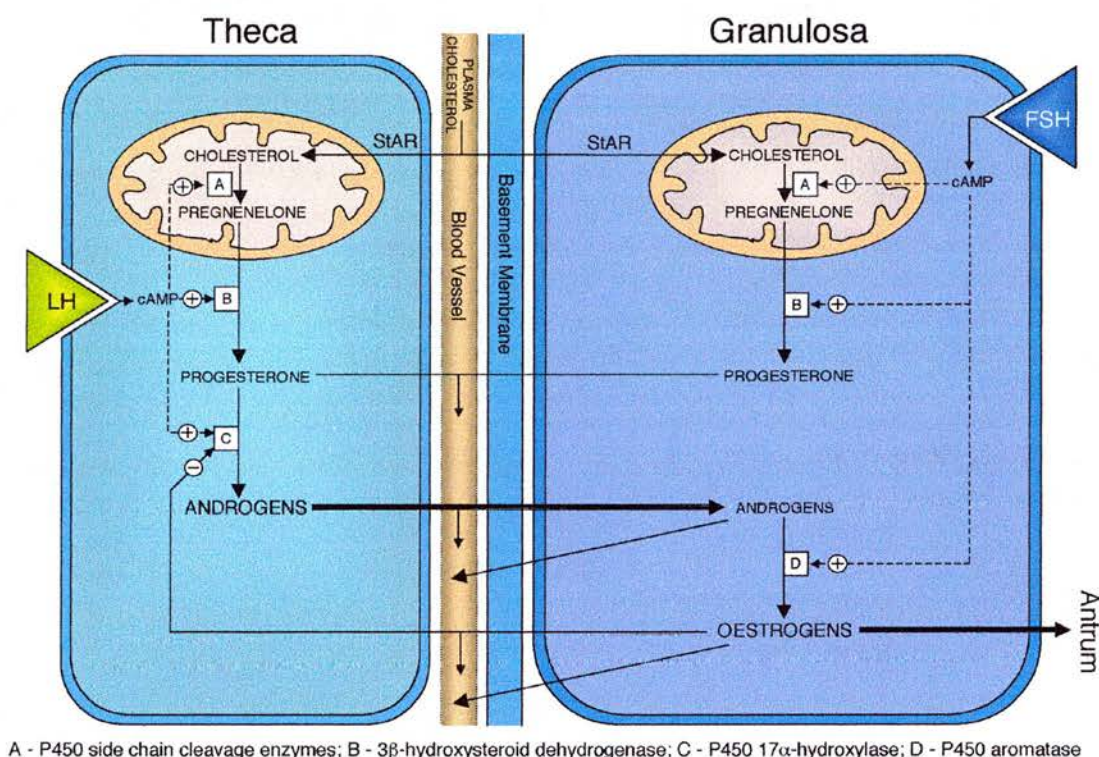


Figure 1.2 The steroidogenic pathway in ovarian follicular cells.

Although the granulosa cells of the ovarian follicle lack the enzymes necessary to produce androgens, they are essential in the conversion of thecal androgen to oestradiol by way of another enzyme, cytochrome P450 aromatase (P450arom). It has been suggested that this reaction occurs mainly in the granulosa cells adjacent to the basement membrane (Baird and McNeilly, 1981), as the androgen substrate on

which P450arom acts is produced by the theca compartment. Aromatase has been shown to be selectively inhibited by follicular fluid from the dominant follicle in sheep (Campbell, *et al.*, 1999a), suggesting the presence of a factor produced by dominant follicles which reduces oestradiol production by other subordinate follicles.

The involvement of both the granulosa and theca compartments in the final production of oestradiol is referred to as the 'two-cell, two-gonadotrophin theory.' In the thecal cells, LH is the primary modulator of P450scc, with FSH in parallel, the controller of P450arom in the granulosa cells (Gore-Langton, 1994; Hillier *et al.*, 1994).

1.4 Oestrous Cycle of the Ewe

After a period of sexual inactivity (anoestrus) in the ewe throughout the summer months, the onset of sexual activity in the autumn is related to the shortening of the photoperiod which is tracked closely by the pineal gland of the animal (Woodfill *et al.*, 1994). The resumption of breeding activity is characterized by a short period of hormonal signalling leading eventually to ovulation (Legan *et al.*, 1977). A dramatic fall in progesterone release brought about by a rise in prostaglandin F-2 α (PGF-2 α) leads to firstly an increase in the level of oestradiol and in turn to a period of sexual receptivity (Barrett *et al.*, 1971), termed oestrus. The feedback of oestradiol to the anterior pituitary brings about a change in the pulse amplitude of LH resulting eventually in ovulation (Scaramuzzi, 1975).

The periods of sexual activity during the autumn and winter months are punctuated with regular cycles of oestrous activity at intervals of approximately 17 days with the day of onset of oestrus designated as day 0 (Figure 1.3). These cycles are normally divided into the luteal phase which lasts from ovulation on day 1, to the regression of the *corpus luteum* (luteolysis) on day 14, and the follicular phase which represents the three day peri-ovulatory period from luteolysis to ovulation (Baird and McNeilly, 1981).

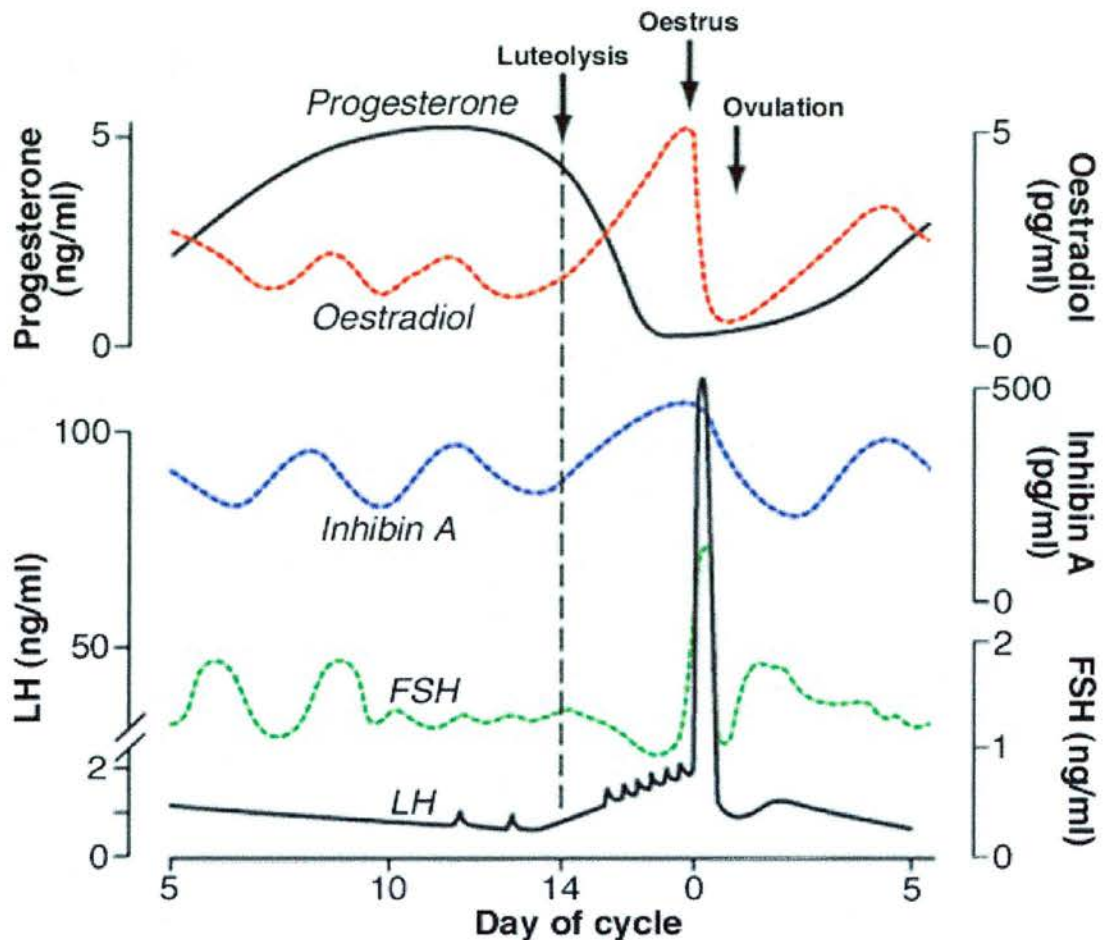


Figure 1.3 The profile of reproductive hormones throughout the ovine oestrous cycle. (Modified from Baird, 1983).

1.4.1 Luteal Phase

The presence of a *corpus luteum* (CL), the post-ovulatory residual mass of follicular cells, in at least one ovary is characteristic of this phase of the ovine cycle. The CL is responsible for the secretion of a high level of progesterone which increases steadily from the time the CL is formed on day 1, to reach a peak between days 6 and 12 (Herriman *et al.*, 1979) (Figure 1.3).

The ovine CL produces almost only progestagens, and in contrast to primates, does not release either oestradiol (Baird *et al.*, 1975) or inhibin (Mann *et al.*, 1989). The main role of progesterone produced by the CL is to prepare the uterine endometrium for implantation and to maintain early pregnancy.

Progesterone secretion by the ovine CL has been shown to be sustained by basal levels of LH in the absence of its pulsatile release (McNeilly and Fraser, 1987). The high level of circulating progesterone in the periphery acts synergistically with oestradiol in a negative feedback loop to reduce LH pulse frequency from the pituitary (Martin *et al.*, 1988), thus enhancing its own release and suppressing ovulation during the lifespan of the CL.

It has been suggested that oestradiol induces an increased sensitivity of the central nervous system to the negative feedback action of progesterone (Goodman *et al.*, 1981a) and that progesterone decreases the frequency of LH pulses from the pituitary by reducing the frequency of GnRH pulses from the hypothalamus (Tamanini *et al.*, 1986).

The decrease in LH pulse frequency leads to a reduction in androgen synthesis in the theca compartment of ovarian follicles and thus to a decrease in oestradiol production by large follicles.

The CL undergoes the process of luteolysis if there is no embryo present in the uterus. The series of events leading to the regression of the CL is primarily controlled by oxytocin, produced by the magnocellular hypothalamic neurons and stored in the pituitary, and prostaglandin F2- α secreted by the uterine endometrium (Alwachi *et al.*, 1981). Progesterone acts to prime the uterus for a period of 7-10 days prior to PGF2- α release by the endometrium (Baird, 1978a). Oxytocin acts on its receptor in the endometrium to stimulate the secretion of PGF2- α which is widely considered to be the main luteolytic agent in the ewe (Umo, 1975; Horton and Poyser, 1976). In addition, the action of PGF2- α on the ovine CL is mediated by the arterial vasoconstrictor peptide, endothelin 1 (Milvae, 2000).

1.4.2 Follicular Phase

During the three day period between luteolysis and ovulation, the ovary is subjected to a continuous rise in LH pulse frequency from the pituitary (Baird, 1978b) due to the fall in plasma progesterone after the regression of the CL (Goodman *et al.*, 1981b), with LH pulse amplitude remaining low (Baird *et al.*, 1981). The immediate effect of the increase in LH release is the induction of the maturation of one or more healthy antral follicles (Baird and McNeilly, 1981). It has been

suggested that progesterone withdrawal is not the only factor responsible for the high LH pulse frequency during this stage of the cycle and that the effect of pulses of oestradiol secreted by the ovary in response to the increasing LH pulses, is also highly significant (Karsch *et al.*, 1983). The level of oestradiol secreted by the ovary eventually reaches a high enough concentration to bring about oestrus behaviour, and triggers the LH surge around 4-8 hours later (Cumming *et al.*, 1973). The surge of LH, lasting between 10-18 hours, induces ovulation approximately 32 hours after the onset of oestrus (Souza, 1994). Ovulatory follicles are derived from large follicles (>2mm) present at the time of luteal regression, however, several reports have shown that if only small follicles are present at this time, the lifespan of the CL may be extended by 24 hours to allow them to develop and ovulate (Tsonis *et al.*, 1984b; Souza *et al.*, 1997a), suggesting that follicle selection in the ewe may occur prior to, or after the regression of the *corpus luteum*.

In addition to the modulation of LH pulses during the follicular phase, the increase in follicular oestradiol and inhibin secretion, primarily from the dominant follicle(s), has a negative effect on the release of FSH by the pituitary (Campbell *et al.*, 1990). Considering recent evidence that FSH modulates early folliculogenesis (Campbell *et al.*, 2000), this reduction in FSH concentration may serve to suppress the recruitment of further follicles and to provide an environment able to select out follicles with the cellular machinery to survive.

1.4.3 Variation in Ovulation rate

The number of ovulations per ovine oestrous cycle, or the ovulation rate (OR), varies considerably from 1 to >10 eggs shed every seventeen days. There are numerous factors which have been shown to affect OR including age (McKenzie, 1937), body weight (Findlay and Cumming, 1976), season (Wheeler *et al.*, 1977) and breed. The sheep is able to vary its OR by extending the length of time to which recruited follicles are exposed to a high enough level of FSH (McNatty and Henderson, 1987) and by the simultaneous maintenance of several follicles able to survive the fall in FSH (Baird and Campbell, 1998), characteristic of the early follicular phase of the cycle. There is also a suggestion that an increase in the

bioavailability of local factors such as IGF-1 (Monniaux *et al.*, 2000) may lead to increased ovarian responsiveness to gonadotrophins.

One of the most prolific ovine phenotypes known is that of the Booroola ewe which evolved from particular lines of the Merino breed in Australia. A gene responsible for the high OR in these animals has been designated the Booroola fecundity (Fec^B) gene (Davis, 1982). Ewes homozygous for this gene have an OR which is more than double that of wild type animals (>5 , compared to 1 or 2), with heterozygotes having an OR of 3 or 4.

This elevation in OR is due to the precocious maturation of small antral follicles which become oestrogenic and express LH receptor on their granulosa cells when they are 2.5-3.5mm in diameter, compared to 4-6mm in wildtype (non-gene carrier) animals (Henderson *et al.*, 1985; McNatty *et al.*, 1986). In addition, it has been shown that there are decreased rates of atresia in large growing follicles of Fec^B carriers compared to wild type animals (Mandiki *et al.*, 2000)

Several reports have shown that the Fec^B gene makes no difference to the hormonal output of the ovary (McNatty, *et al.*, 1986; Souza *et al.*, 1997b), and although ovulatory follicles are smaller in gene carriers there are a greater number leading to an equivalent number of granulosa cells in oestrogenic follicles overall (McNatty *et al.*, 1985). In contrast to ovarian data, a higher frequency of high-level FSH is seen in the plasma of Fec^B animals (McNatty and Henderson, 1987) resulting in an extended period of recruitment. Although these observations indicate some of the differences evident in these animals, the protein for which the Fec^B gene codes, and the precise mechanism of its physiological effects have remained unknown until recently.

The Fec^B gene has been mapped to chromosome 6 in the sheep (Montgomery *et al.*, 1994) and recent studies (Souza *et al.*, 2001; Wilson *et al.*, 2001) have investigated a candidate gene in the same region of the chromosome as the Fec^B locus.

This selected gene codes for the bone morphogenetic protein receptor (BMPR) type 1B also known as activin-like kinase-6 (ALK-6) which binds members of the TGF- β superfamily and initiates gene transcription through the intracellular Smad protein cascade, which will be described later in this review.

Identification of two point mutations highly specific to carriers of the Fec^B

gene in the kinase domain of this receptor (Souza, *et al.*, 2001), suggests that the downstream signalling of the ALK-6 receptor may be disrupted in these animals. This alteration in BMP signalling, essential in mammalian embryogenesis, may well result in the Booroola phenotype. Recent work supporting this hypothesis has studied the response of granulosa cells from animals homozygous for the *Fec^B* gene to ALK-6 ligands. These cells were less responsive to the inhibition of steroidogenesis by GDF-5 and BMP-4 compared to cells from non-carrier ewes (Mulsant *et al.*, 2001) suggesting that the ALK-6 mutation may be responsible for the advanced maturation of ovulatory follicles in *Fec^B* animals.

1.5 Inhibin

Forty years after first being described, numerous studies in the 1970's accumulated evidence for the existence of the non-steroidal hormone, inhibin, and of its ability to suppress FSH secretion by the anterior pituitary (De Jong and Sharpe, 1976; Keogh *et al.*, 1976).

Further research revealed that inhibin is synthesized within the granulosa cells of the mammalian follicle (and luteal cells in primates) (Roberts *et al.*, 1993), either accumulating in the follicular fluid (FF) or being secreted into the plasma. The concentration of inhibin in follicular fluid increases with follicular size and is correlated with aromatase activity in the granulosa cells and in turn the level of oestradiol production (Baird, *et al.*, 1991).

1.5.1 Properties

To date, two forms of inhibin (A and B) have been identified in the ovaries of most species studied, including human, monkey, pig, rat and mouse. They are members of the TGF- β superfamily of heterodimeric glycoproteins and consist of a conserved α -subunit (around 32 kDa) linked by a disulphide bond to one of two β -subunits, β A or β B (14-18 kDa) (Ling *et al.*, 1985). Although β C, β D, and β E subunits have been identified (Hotten, 1995; Oda *et al.*, 1995; Fang *et al.*, 1996), there is little data available describing their physiological effects, with the exception of research indicating that the β E subunit may have a role in liver function and inflammation in the rat (O'Bryan *et al.*, 2000).

Inhibin is synthesized by ovarian granulosa cells as a 105 kDa protein comprised of an α and β subunit, which is then cleaved down into smaller dimers. The α and β subunits are produced independently as large precursor proteins with several processing sites (Forage *et al.*, 1986) (Figure 1.4).

Separate genes code for the α , β A and β B precursors which are processed at regions containing paired arginine residues to produce a 43 kDa α -subunit (α N α C) and a 14-15 kDa β -subunit. The 43 kDa chain is subsequently cleaved to yield a reduced 20 kDa α -chain which is found in 32 kDa inhibin. These steps may occur before or after dimerisation with the β -subunit; however, the fact that contact with serum results in the synthesis of the 32 kDa form (McLachlan *et al.*, 1986) suggests the involvement of a controlling factor in the circulation.

A variety of molecular forms of inhibin have been identified in the human (Sugino *et al.*, 1992; Good *et al.*, 1995; Mason *et al.*, 1996), including mature (32-kDa), partially processed β -precursor dimers and biologically inactive forms of the α -subunit (Knight *et al.*, 1989; Lambert-Messerlian *et al.*, 1994).

Early methods employed in an attempt to measure inhibin levels (Rivier *et al.*, 1986; Sharpe *et al.*, 1988; Campbell, *et al.*, 1990) were targeted against the α -subunit and so captured the biologically inactive free α -subunit forms as well as the biopotent dimers, generating contrasting results. Recent advances have overcome this problem with the development of highly specific two-site enzyme-linked immunosorbent assays (ELISAs) which target the individual β -subunits (Groome *et al.*, 1996; Robertson *et al.*, 1997).

Investigations utilising these new procedures have shown that in primates there is a changing pattern in mRNA expression relating to the inhibin subunits (Fraser *et al.*, 1993). Expression of the β B-subunit is highest in antral follicles and is consistent with the production of activin. In the *corpus luteum*, alpha subunit is predominant over low β -subunit expression and supports the production of inhibin by the *corpus luteum* in the primate.

During the purification of inhibin from follicular fluid, two further molecules (also produced by granulosa cells), termed activin and follistatin (FSP), were identified as additional modulators of pituitary FSH release (Ling *et al.*, 1986; Vale *et*

al., 1986; Robertson *et al.*, 1987). Activin was identified as a disulphide-linked dimer of two inhibin β -subunits (Mr 25,000), existing as either activin A (β A- β A), activin B (β B- β B) or activin AB (β A- β B). Activin stimulates pituitary FSH release and enhances FSH β mRNA expression by stabilising the protein (Carroll *et al.*, 1991b), therefore having the opposite effect to inhibin on the pituitary.

Follistatin is a glycoprotein chain encoded by a single gene with no homology to either inhibin or activin (Shimasaki *et al.*, 1988). Various forms of follistatin with molecular weights between 32 and 39kDa have been identified, each having the capacity to bind to activin, and to a lesser extent, inhibin through their common β -subunits (Nakamura *et al.*, 1990; Shimonaka *et al.*, 1991). Thus, follistatin is able to influence the paracrine/autocrine action of these molecules by modulating their bioavailability (Xiao *et al.*, 1992; Findlay, 1993). It has been shown that FSP production is up-regulated in rat granulosa cells by FSH and activin, possibly through the protein kinase A and C pathways (Shintani *et al.*, 1997).

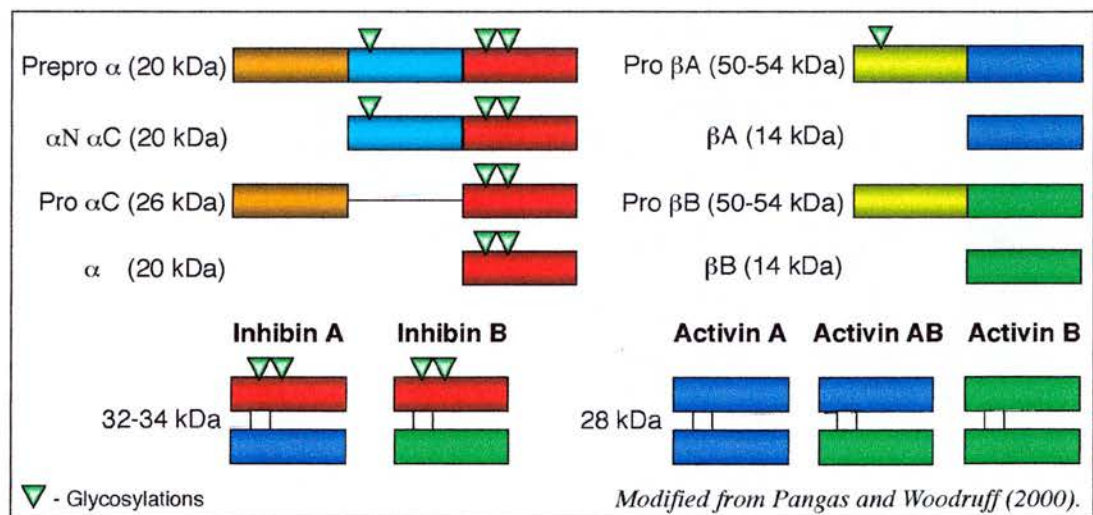


Figure 1.4 Forms of inhibins and activins

In order to learn more about the local and systemic actions of inhibins and activins, it is necessary to review the literature concerning the discovery of their receptors and associated signalling pathways.

1.5.2 Receptors for Inhibins and Activins

A number of the physiological actions of inhibin are directly counteracted by the presence of activin (Ling, *et al.*, 1986), however, the fact that one protein is able to exert effects on tissues in the presence of the other, with no antagonism, suggests the presence of specific receptors and signalling pathways for both hormones.

The first real breakthrough in the search for activin and inhibin receptors came in the early 1990's with the identification and cloning of an activin type II receptor (ActRIIA) which bound activin A and B with high affinity, as well as inhibin A (Mathews and Vale, 1991). The type II activin receptors (ActRIIA and the highly homologous ActRIIB) are serine/threonine kinase receptors with a single transmembrane domain (Attisano and Wrana, 1998; Piek, 1999). Binding of activin ligand to a type II receptor leads to the recruitment of a TGF- β superfamily type I receptor, such as activin-like kinase-4 (ALK4/ActRIB) or activin-like kinase-2 (ALK-2/ActRI) and forms a heteromeric receptor complex (Carcamo *et al.*, 1994). Within this complex, the type II receptor phosphorylates the TGF- β type I receptor on serine and threonine residues within the cytoplasmic domain, close to the transmembrane region, in order to induce activation (Attisano *et al.*, 1996). The activated type I receptor subsequently propagates the signal by phosphorylating downstream proteins, including the Smad (Mothers against DPP) transcription factors (Liu *et al.*, 1996) (Figure 1.5a). Three groups of Smad proteins have been discovered; the receptor Smads, which are phosphorylated by type I receptors; inhibitory Smads which inhibit this process of receptor Smad activation by binding to the type I receptor (Lebrun *et al.*, 1999); and finally the common mediator Smad, Smad 4. Activin and TGF- β ligands signal through the receptor Smads, 2 and 3 (Eppert *et al.*, 1996; Graff *et al.*, 1996; Zhang *et al.*, 1996), with bone morphogenetic proteins (BMPs) signalling through Smad1, Smad5 and Smad8 (Massague and Chen, 2000). The phosphorylation of Smad2 or 3, by the type I receptor after the binding of activin, is modulated by the inhibitory Smad, Smad7 (Lebrun, *et al.*, 1999). Once activated, the receptor Smads (r-Smads) are able to form trimers with Smad 4. These complexes then translocate to the cell nucleus where they induce expression of target genes by binding to promoter elements within the cell's DNA (Derynck, 1998).

Studies incorporating radioactively-labelled recombinant preparations of both inhibin A and activin A revealed specific binding of both proteins to sites in the ovary and testes of the rat (Woodruff *et al.*, 1993; Krummen *et al.*, 1994). Although activin receptors had been identified at this time, receptors for inhibin remained elusive.

Research carried out in parallel to the activin receptor studies identified several factors capable of the high affinity binding of inhibin. Betaglycan, previously regarded as a highly expressed type III TGF- β receptor, was unmasked as one of these inhibin-binding proteins in the late 1990's (Lewis *et al.*, 2000). Cells expressing both betaglycan and ActRIIA were shown to bind inhibin with even greater affinity than betaglycan alone leading to an increase in the antagonism of activin signalling by reducing the bioavailability of ActRIIA receptor (Figure 1.5c).

Further research using an inhibin-A affinity column, identified an inhibin-binding moiety, present in the target tissues of inhibin (Chong *et al.*, 2000). This protein, termed p120 or inhibin binding protein (InhBP) was shown to be a membrane-anchored proteoglycan, expressed concomitantly with FSH β in the pituitary and Leydig cells of the rat. InhBP has no identifiable intracellular serine/threonine kinase domain which distinguishes it from the other TGF- β receptors, and seems to exert intracellular effects by modulating activin receptor complexes (Figure 1.5b).

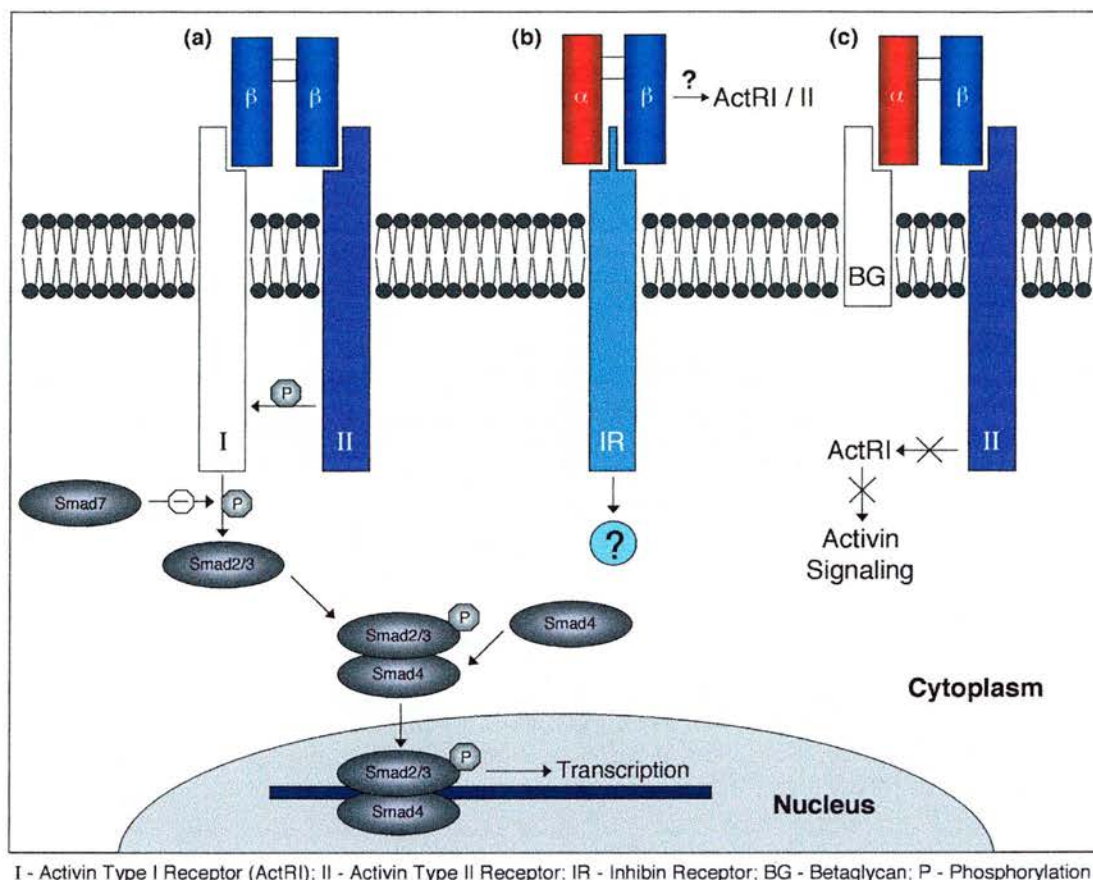


Figure 1.5 Inhibin and activin signalling pathways. (a) Signalling mechanism of activin binding to the type II receptors which recruit and phosphorylate type I receptors. The internal signalling cascade is then initiated by the phosphorylation of Smad2 or 3. (b) Inhibin signalling via inhibin receptor (p120). (c) reduced availability of type II receptor for activin signalling due to recruitment by the betaglycan/inhibin complex.

1.5.3 Mechanism of Action

In all mammals studied, inhibin generates a dose-related suppression of FSH release from the anterior pituitary with a slight suppression of LH levels seen at high concentrations in some species (Farnworth *et al.*, 1988). In contrast to GnRH antagonists, inhibin has the ability to prevent both the preovulatory and secondary FSH surges during the oestrous cycle.

Initially it was thought that inhibin exerted its effect on FSH release by acting directly at the genetic level, by altering the level of FSH β gene expression in the sheep (Mercer *et al.*, 1987). However, further investigations on rat pituitary cells carried out *in vitro*, indicated that inhibin decreases the stability of FSH β mRNA,

with activin increasing its stability to exert its opposing effect (Attardi and Winters, 1993). Inhibin is able to modulate this action of activin in the pituitary by specifically blocking the binding of the molecule to its receptor in Chinese hamster ovary cells (Martens *et al.*, 1997).

Although GnRH is responsible for the regulation of both FSH and LH secretion, inhibin only modulates FSH and has no effect on the expression of LH β mRNA in rat pituitary cells (Carroll *et al.*, 1989). In addition, expression of LH receptor mRNA is controlled by both oestradiol and progesterone in the ewe (Mann *et al.*, 1992).

In addition to their functions at the pituitary, there is considerable evidence implicating inhibin and activins in paracrine and autocrine modulation within the ovary.

1.5.4 Autocrine effects on Granulosa

Inhibin A binding sites have been identified on granulosa cells indicating a likely autocrine action (Woodruff, *et al.*, 1993). In agreement with this finding, an autocrine effect of inhibin on the production of oestradiol by rat granulosa cells has been recorded (Ying *et al.*, 1986) and in cultured sheep granulosa cells inhibin enhances the ability of FSH to induce aromatase (Campbell and Baird, 2001).

Investigations concerning the action of inhibin on granulosa cells are complicated by the production and secretion of endogenous inhibin by the granulosa. This has not, however, been a problem encountered during studies relating to the effects of activin on granulosa cells, which have been well researched in several species, although at this time no evidence is available for the ewe.

Activin has been shown to stimulate the *in vitro* growth of isolated preantral follicles from immature rats, with similar follicles from adult rats showing no response (Yokota *et al.*, 1997).

In isolated rat granulosa cultures, activin is able to up-regulate FSH receptor expression, enhance cellular responsiveness to both FSH and LH (Xiao, *et al.*, 1992) and also increase inhibin α -subunit and FSP mRNA expression (LaPolt *et al.*, 1989; Michel *et al.*, 1992). In the presence of FSH, activin enhances aromatase activity (Miro *et al.*, 1991) and thus oestrogen production as well as progesterone secretion.

These observations indicate that activin may play a significant role in the attainment of FSH responsiveness by granulosa cells (Findlay, 1993), and has a role in the continued development of follicles either responsive or reliant on stimulation via FSH.

Treatment with FSP suppresses FSH-induced aromatase activity, inhibin synthesis and increases FSH-induced progesterone production (Xiao *et al.*, 1990; Xiao and Findlay, 1991). It is important to note that other local ovarian factors including IGF-1 (insulin-like growth factor), EGF (epidermal growth factor) and TGF- α have been shown to affect the growth and differentiation of granulosa cells *in vitro*, as well as the production and release of oestradiol and inhibin A (Campbell *et al.*, 1996a).

1.5.5 Paracrine effects on Theca

In preparations of cultured theca cells from the cow, human and sheep, inhibin enhances LH-induced androgen production (Figure 1.6) with activin having the opposite effect and suppressing this modulation (Hillier *et al.*, 1991a; Wrathall and Knight, 1995; Campbell and Baird, 2001). Incubation of whole (or sections of) follicles with antibodies raised against inhibin, reduced androgen secretion (Smyth *et al.*, 1993). These observations suggest a role for inhibin in the maintenance of thecal androgen production and in turn the supply of androgen substrate for the follicular production of oestrogen in the granulosa. This modulatory effect of inhibin may be significant with respect to oestrogenic follicles in the ovary assuming dominance.

As the dominant follicle emerges, production of inhibin and oestradiol-17 β by the ovary increases (Findlay *et al.*, 1990), thus reducing FSH levels and restricting the growth of other antral follicles. The dominant follicle is able to survive via the action of local factors including inhibin, and IGF-1, which increase the responsiveness of follicular cells to gonadotrophins (Campbell, 1999) thus compensating for the decrease in FSH. In addition the dominant follicles may transfer their dependency from FSH to LH due to the expression of LH receptor on the granulosa cells with LH acting as a surrogate during this stage of the cycle. Subordinate follicles are not equipped with these necessary survival mechanisms (Evans and Fortune, 1997) and become atretic.

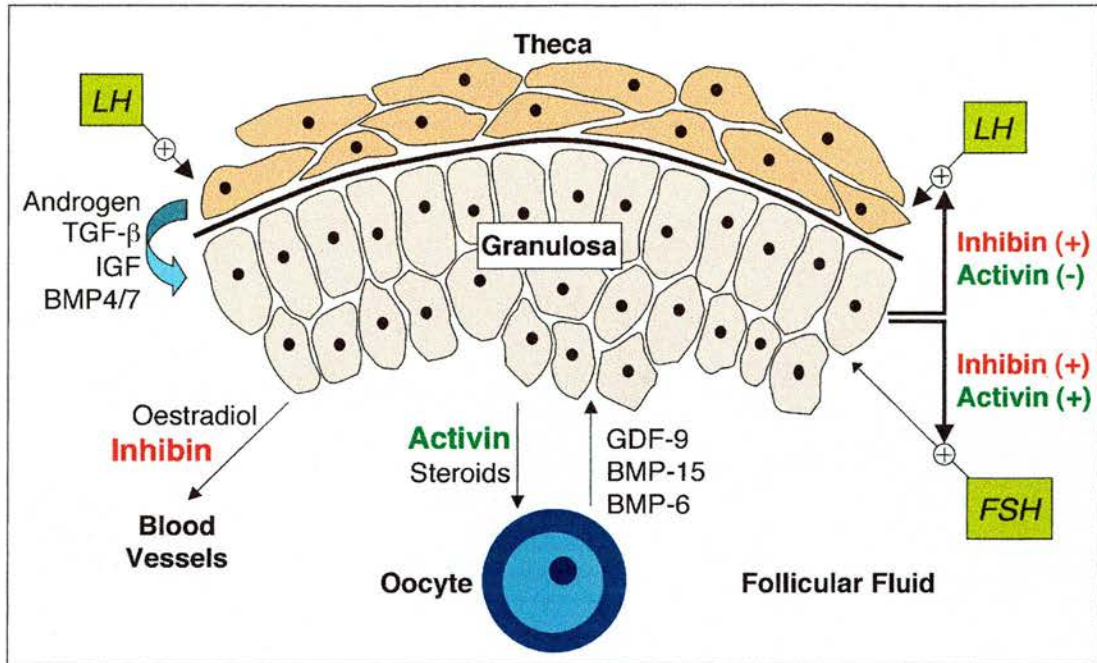


Figure 1.6 Molecular interactions between the compartments of the ovarian follicle.

1.5.6 Paracrine effects on Oocytes

Recent investigation has shown that cumulus granulosa cells express protein and mRNA for all three of the inhibin subunits and FSP during *in vitro* maturation of the oocyte (Izadyar *et al.*, 1998; Silva and Knight, 1998). Expression of activin receptors by oocytes has also been observed (Cameron *et al.*, 1994; Sidis *et al.*, 1998). Inhibin subunit mRNA is only seen in cumulus cells, with expression of inhibin- α and β A mRNA maximal in the cells adjacent to the oocyte (Braw-Tal, 1994). In contrast, inhibin protein was found in both the cumulus and the oocyte (Imai *et al.*, 1996). Furthermore, measurement of activin in oocyte-cumulus complex culture medium revealed that morphologically good quality oocytes lead to the production of higher levels of activin (Lau *et al.*, 1999). These observations suggest the involvement of activin in oocyte maturation *in vivo*, with its action potentiated by inhibin and/or FSP.

1.5.7 Activin Diffusion

Research focusing on the action of activin signalling in the early *Xenopus* embryo has revealed that activin has a long range signalling activity through tissue and does so by forming a concentration gradient by diffusion through the cell layers

(McDowell *et al.*, 1997). By diffusing in this way, activin is able to activate gene transcription in target cells in a concentration-dependent manner, acting like a morphogen (Gurdon *et al.*, 1999). If this is the case in the ovary, the diffusion of activin from follicles may influence the growth and differentiation of other follicles in a morphogen-like manner, with larger follicles having an influence on smaller neighbouring follicles.

1.5.8 Control of Inhibin Secretion from granulosa cells

The release of inhibin is regulated by a large number of factors operating at different levels including endocrine (FSH/LH), paracrine (EGF, TGF- α , INF- γ , androstenedione) and autocrine (IGF-1, TGF- β , activin, FSP) molecules (Tate *et al.*, 1996; Tekmal *et al.*, 1996; Bergh *et al.*, 1997).

The presence of FSH in the follicular environment stimulates the synthesis and secretion of inhibin by up-regulating the expression of mRNA coding for the α and β chains (Rohan *et al.*, 1991). Inhibin production by cultured rat granulosa cells is responsive to both FSH and LH (Suzuki *et al.*, 1987), whilst luteinised human granulosa cells are stimulated by LH alone (Tsonis *et al.*, 1987). In immature human granulosa cells (those from small antral follicles) it is FSH which stimulates inhibin production and not LH (sheep: Campbell and Baird, 2001) with the action of FSH open to modulation by androgen (Hillier *et al.*, 1991b).

These investigations show that the gonadotrophic control of inhibin production varies as the follicle matures. The granulosa cells of small antral follicles do not respond to LH, as they do not possess LH receptors via which inhibin production is enhanced. However, as follicles develop, the expression of LH receptor on the granulosa cells leads to responsiveness to both gonadotrophins.

The presence of pharmacological agents (cAMP, forskolin, phosphodiesterase inhibitors, and prostaglandin E) able to increase the intracellular cAMP concentration within the granulosa all stimulate inhibin production with a dose-response similar to the gonadotrophins (Bicsak *et al.*, 1986; Petraglia *et al.*, 1987), suggesting FSH and LH increase inhibin production via the adenylate cyclase system in conjunction with specific cell-surface receptors.

1.5.9 Molecular weight forms of inhibin and activin

Much of the early work carried out to investigate the *in vivo* action of inhibin focused on the mature 32-kDa form of the protein (Tsafriri *et al.*, 1989; Hillier, *et al.*, 1991b; Schneyer *et al.*, 1991). However, further research into the numerous other molecular forms of inhibin revealed possible roles in follicular development.

Studies in the cow (Sugino, *et al.*, 1992) characterized numerous inhibin forms in follicular fluid, from a high molecular weight (105 kDa) precursor dimer to the mature 32 kDa form of the protein. This research also suggested the serial processing of the 105 kDa form, to smaller 95 and 55 kDa proteins before the final generation of the mature 32 kDa. When added to cultured pituitary cells (Miyamoto *et al.*, 1985), the 32, 55 and 95 kDa forms were equally effective at suppressing FSH release. However, there is a distinct possibility that the heavier forms are converted to the 32 kDa form in the culture system.

Transient mutation of the processing sites on the inhibin precursors (Mason, *et al.*, 1996) leads to the synthesis of non-cleavable varieties of high molecular weight inhibins. Recombinant expression of human α and β A subunit cDNAs in a mammalian cell-line brings about the release of 20-53 kDa products derived from the α subunit, 30-105 kDa dimers of α and β A subunit forms and activin dimers with molecular weights of between 24 and 110 kDa. Following the production of these forms of inhibin and activin, their relative biological activities were analysed. The non-cleavable 55 and 65 kDa forms of inhibin A were found to be fully biopotent in terms of suppressing FSH release from rat pituitary cells. Conversely, the high molecular weight (110 kDa) form of activin A had no measurable effect on FSH release by rat pituitary cells and it is therefore suggested that the processing of this dimer to the 24 kDa form is necessary to render it biologically active.

Further work examining the expression of various mass forms of inhibin by dominant follicles during the luteal and follicular phase in heifers, identified seven distinct proteins in the follicular fluid (Sunderland *et al.*, 1996). The amounts and proportions of these forms varies during atresia and pre and post the LH surge. When considered along with data from *in vitro* studies which show high molecular weight forms exerting effects similar to mature inhibin (Schneyer, *et al.*, 1991; Ghosh, 1994; Padmanabhan *et al.*, 1994), there is a strong suggestion that these molecules

have a significant biological activity.

1.5.10 Inhibin Expression during the Oestrous cycle

To answer questions regarding the involvement of inhibin and its related peptides in the regulation of the mammalian oestrous cycle it is helpful to study the relative levels of the molecules during the considerable physiological and morphological changes occurring in the ovary.

Although both inhibin A and inhibin B are able to suppress FSH release from the pituitary, there is a suggestion that the two molecules have a greater functional significance at different stages of the cycle (Lahlou *et al.*, 1999).

In a number of species studied (including the human) a variation in the expression of mRNA coding for the different inhibin subunits has been shown during the stages of follicle development. In the sheep, mRNAs for the α and β B subunits are evident in the granulosa cells of those follicles which have developed past the primary stage, while β A mRNA is only detectable in healthy antral follicles (McNatty *et al.*, 2000). Research examining the expression of inhibin mRNA during the reproductive cycle showed little change in the production of signal for the proteins (Tisdall *et al.*, 1994). However, other workers (Engelhardt *et al.*, 1993) have reported a suppression of inhibin mRNA shortly after the LH surge, with no notable staining for any subunit in the ovine *corpus luteum*.

In primates the expression of inhibin subunit mRNA is somewhat different, the notable differences being that antral follicles express high level β B subunit mRNA in relation to the other two subunits suggesting the high-level production of activin, and that the primate *corpus luteum* expresses mRNA for all three subunits (Fraser, *et al.*, 1993).

Measuring the levels of immunoreactive (ir) inhibin A and B produced by the expression of these inhibin subunits has, as yet, not been possible in the sheep due to the lack of a suitable assay for inhibin B; it has however, been carried out in other species.

With respect to the levels of ir-inhibin A in the circulation during the human menstrual cycle, levels are low in the early mid-follicular phase, rising in the late follicular phase before falling shortly after ovulation and becoming maximal during the

midluteal phase (McLachlan *et al.*, 1987; Groome *et al.*, 1994). These findings are broadly similar to those recorded employing less specific inhibin assays and suggest that in addition to oestradiol, inhibin A may be responsible for the control of FSH secretion during the luteal phase and the initiation of follicular development during the luteal-follicular transition (Hayes *et al.*, 1998).

The levels of inhibin B in the human, however, do not follow the pattern recorded in earlier assays (Welt *et al.*, 1997). An early rise in its levels suggest that its secretion is induced by the action of FSH on antral follicles, with research showing that the addition of FSH to cultured rat granulosa cells leads to a dose dependent increase in inhibin-B production (Lanuza *et al.*, 1999). However, there is no clear evidence as yet for its involvement in the regulation of FSH during the ovarian cycle.

A similar situation has been noted in the sheep, where it has been shown that during the luteal phase there are three waves of follicular development all preceded by an increase in FSH. The involvement of inhibin A and oestradiol has been described in the control of FSH throughout the first wave of development. As the control during the later waves is unknown and inhibin B has yet to be measured during the ovine cycle, it has been suggested as a putative regulator of FSH release during waves two and three (Souza *et al.*, 1998). Recent studies have shown that contrary to the male human, the ram does not produce inhibin B, and only synthesizes inhibin A (McNeilly, 2000). Thus it is possible that the ewe does not produce inhibin B either and that other factors modulate the later waves of follicle growth.

There are many questions still remaining surrounding the roles of inhibin in all of the species studied. Although recent research has lead to the development of specific assays for the two forms of inhibin, further work is necessary to determine the precise involvement of the glycoproteins in the regulation of folliculogenesis throughout the oestrous cycle.

At the time of writing this review, levels of inhibin A have been measured throughout the ovine oestrous cycle, however, the specific source of the hormone in the ewe has yet to be identified. In addition, the expression of the subunits bound together to produce inhibins and activins has yet to be studied during the reproductive cycle of the sheep. Research in these areas is necessary in order to clarify the contribution of these proteins to the phenomena of follicle development

and selection in this particular species. Furthermore, the involvement of inhibin-B, in the mechanisms of this complicated process has yet to be elucidated, due to the lack of a specific assay for this dimer in the sheep. Further work is required to confirm the suggested influence of this protein in the control of the second and third follicular waves in the ewe.

Although there is much evidence implicating inhibins and activins in the growth and development of ovarian follicles, the possible involvement of these factors in the growth of follicles in breeds of sheep with high ovulation rates has yet to be researched.

Chapter 2 - Materials and Methods

2.1 Experimental Animals

Scottish Blackface ewes used for the experiments in Chapters 3 and 4 of this thesis were housed outdoors at the Marshall Building, Roslin, Mid Lothian, Edinburgh. Additional tissue was obtained from a local abattoir and from in house tissue collections.

2.2 Synchronisation of Oestrous cycles

The animals used during this research were taken from a spontaneously cycling flock and therefore to enable ovariectomy to be carried out at precise points during the oestrous cycle, the ewes were synchronised using medroxyprogesterone acetate sponges (60 mg/sponge, Upjohn Animal Health Division, Crawley, Sussex, UK) followed by i.m. injection of 100 µg cloprostenol, a potent prostaglandin F_{2α} analogue (Estrumate, Cooper's Animal Health Ltd, Crewe, Cheshire, UK).

2.3 Ovariectomy

Anaesthesia was induced by minimal quantities (200-300 mg) of a mixture of two parts thiopentone (Intraval; RMB Animal Health Ltd, Dagenham, Essex, UK) and one part pentobarbitone sodium (Sagatal; May and Baker Ltd, Dagenham, Essex, UK) and maintained using halothane (1.5 – 2.5%). Mid-ventral laparotomy was then performed, the reproductive tract exteriorised and the ovaries removed.

2.4 Blood Sample Collection

2.4.1 Jugular Venous Blood

The left jugular vein was cannulated under local anaesthetic (2ml s.c. of Lignocaine 2%, Lignavet, Leyland, UK) with a 60cm length of Silastic tubing (0.04 x 0.085 inches, internal and external diameters respectively; Osteotec Ltd., Christchurch, Dorset, UK) filled with heparinised saline (10 IU/ml), which was inserted into the

jugular vein through the bore of a suitably sized needle to a depth of 10cm and a 10ml sample extracted.

2.4.2 Timed Ovarian vein Samples

In the 18 animals designated for follicle dissection, both utero-ovarian veins were cannulated at the uterine branch with silastic tubing (1.6 ID x 3.2mm OD, Don Corning, Mid Law, USA). The end of the tubing was then advanced so it lay within the utero-ovarian vein and the uterine branch tied off with a suture. The proximal end of the ovarian vein was then occluded and a timed 10ml blood sample taken from each side of the tract.

2.5 Follicle Dissection and Incubation

Isolated ovaries were microdissected using sterilised forceps with each antral follicle recovered being placed into a petri dish containing M199 medium (Sigma, Dorset, UK), previously warmed to 37°C. After the dissection of all visible antral follicles from an ovary, the diameter of each follicle was measured and recorded by placing the petri dish onto a measuring grid under a microscope. Immediately after measuring the follicles each individual follicle was transferred to a separate well of a 24 well culture plate, with each well containing 1ml of M199 (37°C). The plates were then incubated at 37°C (5% CO₂) for 2 hours, after which each follicle was transferred to a well containing a fresh 1ml of M199 on a new culture plate. The incubation plates were then covered with adhesive plastic covers (Sigma) and stored at -25°C for assay. After transfer to the new plates the follicles were hemisected under a microscope using microscissors with half of the follicle shell fixed in 4% paraformaldehyde, and the other half snap frozen in liquid nitrogen. The plates containing the follicular fluids in M199 were immediately frozen at -25°C for assay.

2.6 Immunoassays

2.6.1 Oestradiol Assay

Oestradiol was measured using an assay method previously described (Baird, *et al.*, 1981) with a sheep anti-oestradiol antibody (BW 26/9/82) used at a final working

dilution of 1:500,000 as first antibody. This assay was run using ^{125}I labelled oestradiol-17 β as a tracer (in house) and oestradiol-17 β (Sigma) as a standard for the assay, in the range 39-10,000 pg/ml. Sensitivity of this assay was 42 pg/ml and the intra and inter assay coefficients of variation were 7.9% and 12.7%. The protocol for this assay can be found in the appendix at the end of this thesis.

2.6.2 Androstenedione Assay

Androstenedione was measured in media samples using a previously described protocol (Campbell, *et al.*, 1990) which employs an anti-androstenedione C3 antibody (Thomson, *et al.*, 1989) at a final working dilution of 1:3,000,000. ^{125}I androstenedione tracer (Donated by the University of Glasgow) and powdered androstenedione (Sigma) as a standard for the assay with a range from 39-10,000 pg/ml. Sensitivity of this assay was 40 pg/ml and the intra and inter assay coefficients of variation were 8.6% and 14.3%. The protocol for this assay may be found in the appendix at the end of this thesis.

2.6.3 Progesterone Assay

Progesterone was measured in unextracted blood samples taken from the jugular veins of the animals studied using a specific radioimmunoassay. This assay was modified to allow the measurement of progesterone in the plasma without extraction (McNeilly and Fraser, 1987). Buffer pH was reduced to 6.0 and 8-Anilino-1 naphthalene sulphonic acid was included to prevent binding of the steroid by carrier proteins present in the blood. The primary antibody used was a lyophilised antiserum (SAPU S235-201) at a working dilution of 1:30,000. Standards were generated in house from purified progesterone (Sigma, Dorset, UK), diluted in absolute ethanol between in the range 1 - 120 nmol/l. The tracer used was ^{125}I labelled progesterone-11 α -glucuronide (Amersham Pharmacia Biotech, Bucks, UK). Sensitivity of this assay was 0.6 nmol/l and the intra and inter assay coefficients of variation for three quality control pools were 6.6% and 7.6%. The protocol for this assay may be found in the appendix at the end of this thesis.

2.6.4 FSH Assay

The measurement of FSH in the peripheral plasma of ewes was carried out using a specific radioimmunoassay (Campbell, *et al*, 1989). The primary antibody used for FSH capture was NIDDK-NIH anti-ovine FSH-1 used at a working dilution of 1:12000. USDA-oFSH-SIAFP-RP-2 was used as a standard (range = 0.1-25 ng/ml) with NIAMMD oFSH-19, iodinated in house via the lactoperoxidase method, used as a tracer. The sensitivity of this assay was 0.1 ng/ml and the intra and inter assay coefficients of variation were 6.6% and 11.8%. The detailed protocol for this assay can be found in the appendix of this thesis.

2.6.5 Inhibin A Assay

Levels of inhibin A were measured using a specific two-site enzyme-linked immunosorbent assay (ELISA) developed for the measurement of the hormone in human biological fluids (Groome, *et al.*, 1994) and modified to detect the ovine form of the dimer (O'Brien, *et al*, 1996). This protocol utilises an antibody sandwich method with one antibody binding inhibin alpha subunit, and the other antibody binding inhibin β A subunit. Detection of inhibin A was carried out using an immobilised mouse monoclonal antibody (E4 clone – N. Groome, Oxford Brookes University, UK) targeted against the β A subunit and a biotinylated alpha C specific PPG monoclonal antibody. Purified 32 kDa bovine inhibin diluted in ovariectomised (OVX) sheep plasma was used as a standard in the range 15 – 1000 pg/ml. Standards, samples and quality controls were boiled and treated with hydrogen peroxide to denature and oxidise the sample and allow antibody binding. The sensitivity of this assay was 15.6 pg/ml and the intra and inter assay coefficients of variation were 3.8% and 4.8%. The full protocol for this assay may be found in the appendix at the end of this thesis.

2.6.6 RIA Counting and Data Reduction

The level of radiation in the assay tubes generated by the radioimmunoassays employed in this work were counted using a Multigamma 1261 counter (Wallac, Turku, Finland) with well-type aluminium-covered sodium iodide crystals. This counter had a minimum efficiency of 75% for the detection of ^{125}I . The data produced

by the gamma counter was then interpreted using AssayZap software (Elsevier Biosoft, Cambridge, UK) which plots standard curves and continually updates intra and inter assay coefficients of variation for each assay protocol.

The 96-well plates used for the detection of inhibin A were interpreted using a LabSystems Multiscan MCC/340 (Life Sciences International Ltd., Basingstoke, UK) plate reader at a wavelength of 492 nm and the results transferred to the AssayZap program as with the radioimmunoassay data.

2.6.7 Statistical Analysis

Statistical tests on the data generated by the work contained in this thesis were carried out using an Apple Macintosh Computer. Student *t*-tests were performed using the Analysis Toolpak plugin found in Microsoft Excel 2001, while analysis of variance followed by Bonferroni's post hoc analysis was carried out using XLSTAT statistical analysis software (AddinSoft, Paris, France).

Further details of statistical tests used can be found in the experimental chapters of this thesis (Chapters 3 – 7).

2.7 Tissue fixation and processing

2.7.1 Fixation of tissue for Paraffin Embedding

Ovarian tissue was placed into 4% paraformaldehyde (PFA) immediately following ovariectomy. The tissue was allowed to fix in this solution for approximately 24 hours before transfer to 70% ethanol until embedded into paraffin wax.

2.7.2 Tissue processing and sectioning

Ovarian tissue was dehydrated by treatment with a series of alcohols prior to the saturation of the samples with paraffin wax. This whole process was carried out using a 17.5 hour automated protocol on a Leica TP-1050 processor (Leica UK Ltd., Milton Keynes, UK). The tissue was then embedded in the desired orientation in molten paraffin wax and the wax allowed to cool prior to the sectioning of the tissue. 5µm sections of the tissue were cut using a hand-operated microtome (Jung RM2035; Leica UK Ltd) and floated on the surface of distilled water (RNase free for *in situ*

hybridisation slides) in a water bath at 40°C. Individual sections were then positioned onto single Superfrost microscope slides (BDH Laboratory Supplies, Dorset, UK) and dried overnight prior to the performance of staining protocols.

2.8 Immunohistochemistry

2.8.1 Antibodies

For detection of the α -subunit, a rabbit polyclonal antibody (R150) raised against amino acids 1-26 of the N-terminus of the porcine inhibin α -subunit conjugated to ovalbumin (Professor A. S. McNeilly, HRSU, Edinburgh, UK) was employed.

Pig α	staplpwpwspaalrllqrppeepavhadchraslnisfqelgwdrwivhppsifihychggcglpt
	--o-----o-----o-----o-----
Sheep α	stpplpwpwspaalrllqrppeepaahadchraalnisfqelgwdrwivhppsififychggcglpt
Pig α	lpnlplsvpgapptpvqplllvpgaqccaalpgtmrslrvrttsdggysfkyetvpnlltqhacaci
	-oo--o--o--o--o--o-----o-----o-----
Sheep α	lqdlplpvpgvpptpfqplslvpgaqaccaalpgtmrplrvrttsdggysfkyemvpnlltqhacaci
Human β A	glecdgkvnicckkqffvsfkdigwndwiiapsgyhanycegecpshiagtsgsslsfhstvinhy
	-----o-----
Sheep β A	glecdgkvnicckkqfyvsfkdigwndwiiapsgyhanycegecpshiagtsgsslsfhstvinhy
Human β A	rmrghspfanlkscvptklrpmsmlyddgqniikkdiqnmimeecgcs

Sheep β A	rmrghspfanlkscvptklrpmsmlyddgqniikkdiqnmimeecgcs
Human β B	glecdgrtnlccrqqqffidfrligwndwiiaptgyygnycegscpaylagvpgsassfhtavnqy
	-----o-----
Sheep β B	glecdgrtslccrqqqffidfrligwndwiiaptgyygnycegscpaylagvpgsassfhtavnqy
Human β B	rmrglnpgtvnscsciptklstmsmlyfddeynivkrdvpnmiveecgca
	-----o--
Sheep β B	rmrglnpgtvnscsciptklstmsmlyfddeynivkrdvpnmiveecaca

Figure 2.1 A comparison of the amino acid sequences of the sheep inhibin subunits with the porcine (α subunit) and human (β subunits).

Mouse monoclonal antibodies raised against amino acids 82-114 of the β A subunit of human inhibin (E4 clone) and amino acids 82-114 of the human β B subunit (12/13 clone), were supplied by Professor Nigel Groome, Oxford Brookes University, UK. The polyclonal anti-Mullerian hormone (AMH) antibody raised in the rabbit was supplied by Dr Nathalie Josso (INSERM U493, France). Figure 2.1 shows the sequence homology between the published ovine inhibin subunit sequences and the amino acid sequences of the porcine and human subunits against which the antibodies employed in this work were raised.

2.8.2 Double Fluorescent Immunohistochemistry

Paraffin wax was removed from the sections by treatment with both xylene and histoclear for five minutes prior to the rehydration of the tissue using a series of alcohols (100 – 70%). Antigen retrieval was carried out by boiling slides in citrate buffer for 5 minutes, after which they were washed twice in phosphate buffered saline (PBS) for five minutes.

Slides were then treated with a blocking solution of normal goat serum diluted 1:5 in PBS for 30 minutes, before being washed as before. Primary antibodies were diluted to the required concentrations in blocking solution and applied to the tissues, with control slides being treated with serum from the same species the primary antibodies were raised in.

The slides were then incubated overnight at 4°C to allow antibody binding. The following day, slides were washed thoroughly in PBS prior to the addition of fluorescent second antibodies (FITC anti-mouse, TRITC anti-rabbit; Sigma, Dorset, UK). These antibodies were diluted to the working dilution of 1:50 in blocking solution and the slides were then left in a hydrated slide box for 1 hour at room temperature. The slides were then washed in PBS before being mounted using aqueous Citifluor mounting solution (Citifluor Ltd., London, UK).

In order to ensure that the antigen retrieval step of this protocol and the fixative used made no significant difference to the immunostaining pattern seen in the ovarian sections the procedure was carried out using ovaries fixed using two preparations commonly used in the fixing of histological tissues and with sections either subjected to the antigen retrieval treatment or not. The results, which are summarised in Table

2.1, show that the variations in the staining method made no difference to the staining pattern observed using the three inhibin antibodies.

	Stage of Follicle	Fixative		Antigen Retrieval	
		PFA	Bouin's	+	-
α Subunit	Pri	-	-	-	-
	SP	-	-	-	-
	LP	+	+	+	+
	SA	+	+	+	+
	MA	++	++	++	++
	LA	++	++	++	++
βA Subunit	Pri	+	+	+	+
	SP	+	+	+	+
	LP	+	+	+	+
	SA	+	+	+	+
	MA	++	++	++	++
	LA	++	++	++	++
βB Subunit	Pri	+	+	+	+
	SP	+	+	+	+
	LP	++	++	++	++
	SA	++	++	++	++
	MA	++	++	++	++
	LA	++	++	++	++

Table 2.1 Effect of tissue fixative and antigen retrieval on the pattern of inhibin subunit immunostaining in the ovine ovary. Pri – Primary; SP – Small preantral; LP – Large Preantral; SA – Small antral; MA – Medium antral; LA – Large antral. Positive staining is indicated by + and strong staining by ++.

2.8.3 Image Analysis

Tissue sections generated by the double fluorescent immunohistochemistry protocol were studied using a Zeiss 510 Laser scanning confocal microscope (Zeiss) which has the capability to detect two wavelengths of fluorescence from the same tissue section, providing two differing fluorescent antibodies have been used for detection. Images were captured using LSM 510 software (Zeiss), which also provided dimensions of the selected area.

For additional image analysis, these images were transferred to an Apple Macintosh computer and imported into Scion Image Analysis software (version 1.62c; Scion Corp., Maryland, USA). This program allows the measurement of pixel

intensity on a scale of 1 (black) to 100 (white) across a selected area. The levels of staining were quantified using this facet of the software to measure the average intensity of fluorescent staining in a consistent region of cells in the tissue sections.

Levels of staining measured in the stromal tissue adjacent to follicles were subtracted from the levels detected in follicular cells to account for non-specific staining. The mean values for each cellular compartment within each size class of follicle were then calculated and plotted. Image montages were created using Adobe Photoshop 6.0 (Adobe Systems Inc., California, USA).

2.8.4 Image Analysis Validation

The method used to quantify levels of fluorescent staining in the tissues described in this thesis is a novel protocol and therefore a form of validation was required for this procedure to ensure low-level inter-experimental variation in the levels of staining recorded.

To provide an indication of the variation in staining measured between slides, 8 consecutive sections were taken from one of the ovaries described in Chapter 3 of this thesis, with all sections then subjected to the series of incubations described in section 2.8.2 of this chapter, with an primary antibody raised against inhibin β B subunit. Image analysis software was then used to produce a graphical profile of the staining detected in cross-sections of two follicles, for each of the serial sections treated. These profiles were plotted for the 8 sections of both an early antral follicle (Figure 2.2a) and a large antral follicle (Figure 2.2b) from the chosen ovary.

These profiles were highly comparable from one section to another indicating a relatively low level of variation between different sections of the same follicles using this system.

In addition, individual measurements were taken from the different cell types in each of the follicle sections using a consistent area of interest, as described in section 2.8.3 of this chapter. These mean pixel densities for each of these compartments from each section of the follicles were then pooled together. The level of staining observed in the stromal cells adjacent to the follicles in each section was

then subtracted from the level measured in the granulosa and theca layers and the standard error for each cell type was calculated for both follicles (Figure 2.3)

The standard errors calculated for the mean pixel densities for each compartment in both follicles studied were very low indicating a high level of consistency in the measurement of staining intensity in follicle compartments using this analysis system.

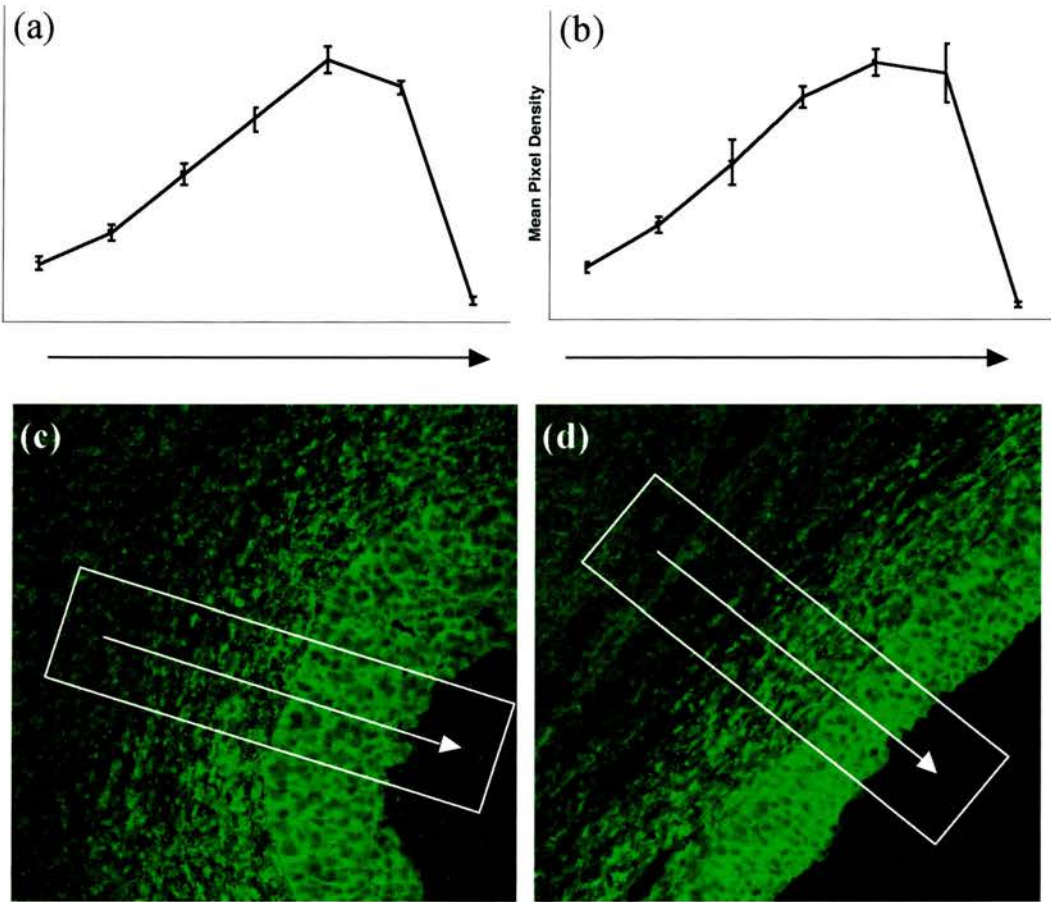


Figure 2.2 Mean Immunostaining profiles for an early antral (a) and a large antral (b) follicle incubated with an inhibin β B subunit antibody (from 8 replicates). Each point is shown \pm SEM. (c) and (d) are representative images (from an early antral follicle and a large antral follicle respectively) from which these profiles were recorded.

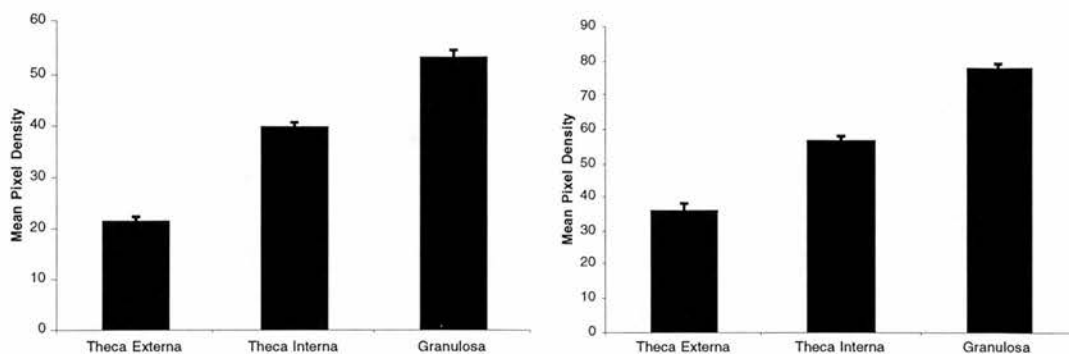


Figure 2.3 Mean levels of inhibin β B staining observed in the cellular compartments of an early antral (a) and large antral (b) follicle.

2.9 Western Blotting

2.9.1 Protein Extraction and Electrophoresis

Protein was extracted from ovine ovarian structures prior to Western blotting. The follicles and additional structures were microdissected from ovaries under sterile conditions and immediately placed into M199 medium (37°C). Tissues were then shredded individually in Falcon tubes containing 1.0 ml protein extraction buffer using a hand held homogeniser.

Follicular fluid samples were collected directly from follicles hemisected in a sterile petri dish and diluted in known volumes of PBS. Protein concentration was estimated using a Genequant (Amersham Pharmacia Biotech) and for each sample at least three measurements were made to ensure reliable readings. In addition, dilutions of the samples were made and read in the same fashion to confirm the original concentrations. The samples were then diluted in protein extraction buffer to give final concentrations of 2 μ g/ μ l. 20 μ l of each sample was then combined with 5 μ l of 5X sample application buffer containing β -mercaptoethanol (reducing) or not (non-reducing).

Samples were separated by electrophoresis along with prestained low range protein markers (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) and the resulting gel electroblotted using a wet transfer method onto PVDF membrane (Amersham Pharmacia Biotech). After the transfer of protein to the membrane, the gel was stained with Coomassie blue solution for 5 minutes.

2.9.2 ECF Detection

Membranes produced by the electroblotting protocol described in the previous section were immediately treated with blocking solution (5% normal swine serum diluted in a Tris-buffered saline/Tween solution) for at least 1 hour at room temperature (or alternatively overnight at 4°C). Membranes were then incubated with primary antibodies diluted to the desired concentration in blocking solution for 1 hour at room temperature and washed thoroughly in TBS-T prior to further incubation with an associated alkaline phosphatase linked second antibody (diluted in blocking solution) for 1 hour at room temperature. After another series of TBS-T washes the membranes were transferred to square petri dishes where they were immersed in enhanced chemifluorescence substrate (ECF; Amersham Pharmacia Biotech) in the dark for 20 minutes. Membranes were then air-dried on a sheet of Whatman paper (Inverclyde Biologicals Ltd., Belshill, UK) for a further 20 minutes in the dark, prior to detection on a Storm 860 Phosphoimager (Amersham Pharmacia Biotech). Images were then transferred to an Apple Macintosh computer and images compiled using Adobe Photoshop 6.0.

2.10 *In situ* Hybridisation

2.10.1 Probe Preparation

Plasmids with inhibin subunit cDNA inserts were linearised (2.5µl plasmid preparation, 2µl specific restriction enzyme, 5µl 5X restriction enzyme buffer (Sigma) and 40.5µl nuclease free water, incubated at 37°C for at least two hours). The restriction enzymes used varied depending on the plasmid being linearised. 2µl of the reaction solution was then run on a 1% agarose gel together with 1µl of uncut plasmid, in order to confirm complete cleavage of the plasmid DNA.

2.10.2 Probe labelling

Riboprobes were created by incubating 1µl of linearised plasmid with: transcription buffer (Promega UK, Southampton, UK), DTT, rRNasin (Sigma), rNTPs, ^{35S} labelled UTP and the appropriate RNA polymerase (T3 or T7) depending

on the plasmid used, for 60 minutes at 37°C. At this point tRNA (Sigma) and DNase were added along with additional transcription buffer and rRNasin. 1µl of each sample was removed prior to a further 15 minute incubation at 37°C, to allow calculation of specific activity for each probe. After incubation the samples were run through RNase spin columns (Amersham Pharmacia Biotech) to remove unincorporated nucleotides and a 1µl volume from each filtered sample was added to 4ml scintillation fluid and read on a β-radiation counter. The amount of radioactivity measured in each sample was noted and the percentage incorporation for each probe was determined. From these data, the quantity of each probe required to give 500,000 cpm per slide was calculated.

2.10.3 Pre-hybridisation

The detailed protocol for this procedure may be found in the appendix at the end of this thesis.

Tissue sections mounted on slides under RNase free conditions were rehydrated by passing through an alcohol gradient (100% - 30%). The slides were then treated with 0.85% NaCl (5 minutes) and 4% paraformaldehyde (20 minutes) with 5 minutes washes with phosphate buffered saline (PBS) after each treatment. A buffered solution (10mM EDTA, 0.1M Tris) of 2µg/ml Proteinase K (Sigma) was added to the slides and incubated for 7.5 minutes at 37°C. Further incubations with 4% PFA (5 minutes) and 2X SSC (2 minutes) were followed by treatment with 0.25% acetic anhydride in 0.1M triethanolamine-HCl for 10 minutes in order to lessen ionic charges in the tissue. The slides were then treated with 2X SSC (2 minutes), dehydrated via an alcohol gradient (60 – 100% Ethanol) and then treated with chloroform (5 minutes) and 100% ethanol (2 minutes) prior to being left to air-dry.

2.10.4 Hybridisation

A volume of ³⁵S labelled probe diluted in hybridisation buffer and calculated to contain 500,000 cpm was added to each slide and the slides incubated in a sealed chamber humidified with 50% formamide in 1X SSC. Parafilm coverslips were placed

onto each slide to ensure complete coverage of the probe over the tissue and the slides incubated at 55°C for 20 hours.

2.10.5 Post Hybridisation

Parafilm was removed from each slide after hybridisation and the slides rinsed thoroughly in 2X SSC (55°C) and then treated with 20mg/ml RNaseA (Sigma) diluted in 2X SSC at 37°C for 1 hour. A series of treatments were then carried out at 55°C with varying concentrations of SSC, formamide and β -mercaptoethanol. Following these treatments the slides were dehydrated through an alcohol gradient (60 – 100% ethanol) and allowed to air-dry.

2.10.6 *In situ* Development

Slides were then coated with Ilford K2 Emulsion (Ilford Ltd., Cheshire, UK) and placed in a sealed lightproof box for at least two hours. Once thoroughly dry, the slides were transferred to another lightproof box and exposed at 4°C for approximately 4 weeks. The slides were developed and fixed in a darkroom by treatment with Kodak D-19 developer (Kodak) for 2.5 minutes, a wash with purified water for 30 seconds and HYPAM fixer for 3 minutes. Finally the slides were rinsed with distilled water in light for 10-15 minutes before being counterstained with haematoxylin and mounted.

Slides were examined using a Nikon Eclipse E800 microscope (Nikon UK Ltd., London, UK) fitted with a dark field condenser and images captured using Scion Image analysis software (Scion Corp).

Chapter 3 - Source of Inhibin-A throughout the Ovine Oestrous Cycle

3.1 Introduction

The ovulatory follicle is selected during the follicular phase of the ovine oestrous cycle. The majority of the recruited antral follicles in the ovary are unable to survive the decline in FSH, which characterises this phase of the cycle, and become atretic. Those follicles able to generate a trophic cAMP response to both FSH and LH make use of the increase in LH pulse frequency in the early follicular phase and avoid atresia. Of these selected follicles the ovulatory follicle is identified as a large, highly visualised follicle secreting a high level of oestradiol (Baird, *et al.*, 1991).

Inhibin and oestradiol are able to inhibit selectively FSH secretion by the anterior pituitary (Mann, *et al.*, 1992) and levels of both of these hormones peak during the follicular phase of the oestrous cycle. As it is the follicles themselves which feedback to the pituitary to maintain the hormonal environment, knowledge of the follicular origin of inhibin A is essential to our understanding of the process of follicle selection.

Previous work, utilising a non-specific assay targeted against all alpha subunit containing factors, and thus detecting non-bioactive as well as bioactive molecules, has indicated the origin of inhibins to be large oestrogenic follicles, with smaller antral follicles also secreting considerable amounts (Campbell *et al.*, 1991; Mann, *et al.*, 1992). The use of a more refined enzyme-linked immunosorbent assay (ELISA) which employs a sandwich method utilising two antibodies, specific for the α and β A subunits has revealed peaks in plasma inhibin-A during both the follicular and early luteal phases (Souza, *et al.*, 1997b; Knight, *et al.*, 1998).

Although this research examined the levels of inhibin A in the circulation during the oestrous cycle, the source of the dimer and any shifts in production and/or release which may occur have yet to be elucidated.

In this study we have used this specific sandwich assay to investigate the origin of inhibin A in the ovine ovary throughout the oestrous cycle by measuring the concentration of the hormone in peripheral (jugular) and ovarian venous blood as well as in the follicular fluids of dissected antral follicles. In addition, the release of inhibin

A, oestradiol and androstenedione from dissected antral follicles during a 2 hour incubation *in vitro* was measured.

3.2 Methods

3.2.1 Experimental Animals

The following work was carried out on 24 Scottish Blackface ewes during the breeding season throughout the period October to December 1998 at the Marshall Building, University of Edinburgh, Lothian.

Oestrous cycles were synchronised using medroxyprogesterone acetate sponges (60 mg/sponge, Upjohn Animal Health Division, Crawley, Sussex, UK) followed by i.m. injection of 100µg cloprostenol, a potent prostaglandin F_{2α} analogue (Estrumate, Cooper's Animal Health Ltd, Crewe, Cheshire, UK). The synchronised ewes were then allocated to one of three groups of eight animals and underwent surgery at one of three time points during the oestrous cycle: late follicular phase (day 1 – oestrus +24h), mid-luteal (day 10), and early in the follicular phase (day 15 – PG +24h). These time points for ovariectomy were selected to provide ovaries containing follicles at different stages of development. Two animals in each group of eight were selected for ovariectomy for the purpose of histological analysis with the other 6 animals having their ovaries removed for follicle dissection.

3.2.2 Experimental Procedure

Ovariectomy and the removal of jugular and timed ovarian vein blood samples was carried out on all animals as previously described in Chapter 2 (2.3 – 2.4.2)

The ovaries were then removed from all animals, with both ovaries from two animals in each group fixed for histological analysis. Ovaries from the remaining 18 sheep were microdissected and all visible antral follicles collected. Follicle diameter was recorded (in mm) prior to each follicle being placed in an individual well of a 24 well culture plate and incubated for 2 hours in 1 ml of Medium 199 (Sigma, Poole, Dorset, UK). Following incubation, each follicle was transferred to a well on a new culture plate containing a fresh 1ml of M199. All follicles were hemisected using microscissors and both sets of medium were frozen at –20°C. Finally, one half of the

follicle shell was fixed in paraformaldehyde and the other half snap frozen in liquid nitrogen.

Follicular fluid volumes were calculated by entering the diameter of each follicle into the following equation:

$$\text{volume} = 0.52(\text{diameter}^{2.7}) \text{ (Carson, } et al., 1981\text{)}.$$

3.2.3 Assays

All resulting media samples from both the incubations and the hemisections were assayed for oestradiol, androstenedione and inhibin A. Ovarian venous blood samples were assayed for oestradiol, progesterone, inhibin A and jugular venous blood samples assayed for LH, inhibin A and progesterone.

Protocols for each of these assays are included in this thesis (Chapter 2 and Appendix).

3.2.4 Analysis of Data

Follicles were classified as small (<3mm) and large (>3mm), with the large follicles further subdivided into non-oestrogenic and oestrogenic follicles. Large, 'healthy' oestrogenic follicles were defined as having a follicular fluid concentration of oestradiol greater than 100ng/ml, a follicular fluid oestradiol: androstenedione ratio greater than 50, and were highly visualised. Linear relationships between venous concentrations of hormones and hormone levels compared with follicle diameter were assessed by regression analysis, while comparisons between levels of hormone release and follicular fluid concentrations were carried out using analysis of variance with post-hoc analysis by Bonferroni's test.

3.3 Results

3.3.1 Venous inhibin A measurements

Blood was collected from the jugular and both ovarian veins of each of the 18 animals selected for follicle dissection. The concentrations of inhibin A in the venous plasma were measured and the correlation between the mean levels of the hormone in the ovarian veins plotted against the jugular level for each animal (Figure 3.1).

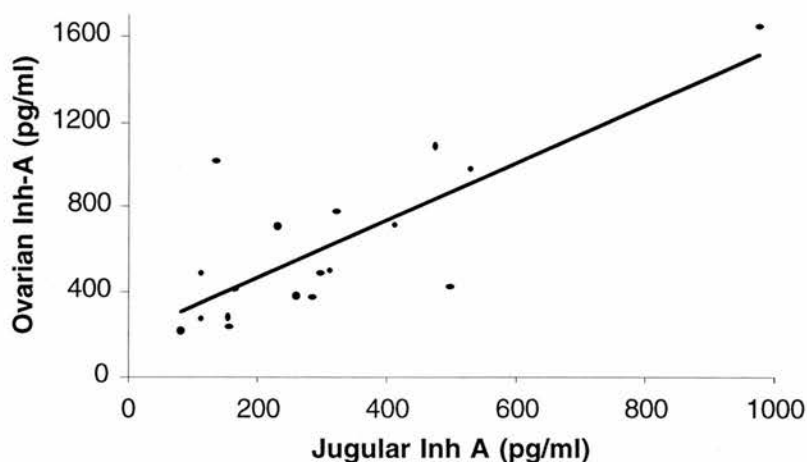


Figure 3.1 Relationship between inhibin A concentration in the ovarian and jugular vein ($n = 18$; $r = 0.782$; $P < 0.001$).

There was a significant positive relationship between the concentrations in the two veins ($r = 0.782$; $P < 0.001$), with the level of inhibin A found in ovarian venous plasma being significantly higher ($P = 0.005$) than in peripheral blood (595 ± 69.2 vs. 281.4 ± 30.6 pg/ml). The concentration of inhibin A was consistently higher in those veins draining an ovary containing a large estrogenic follicle than those without ($P < 0.001$) (Figure 3.2). Although the level of inhibin A was higher in the ovarian vein than in the jugular when including all experimental animals, when divided into the three stages of the cycle, the only significant difference was during the mid luteal phase (Figure 3.3). There was no significant variation in inhibin A concentration in either vein during the cycle, although the results suggest a decrease in inhibin A release during the follicular phase. It is probable that the small number of measurements for each group explains the lack of significant differences between these time points.

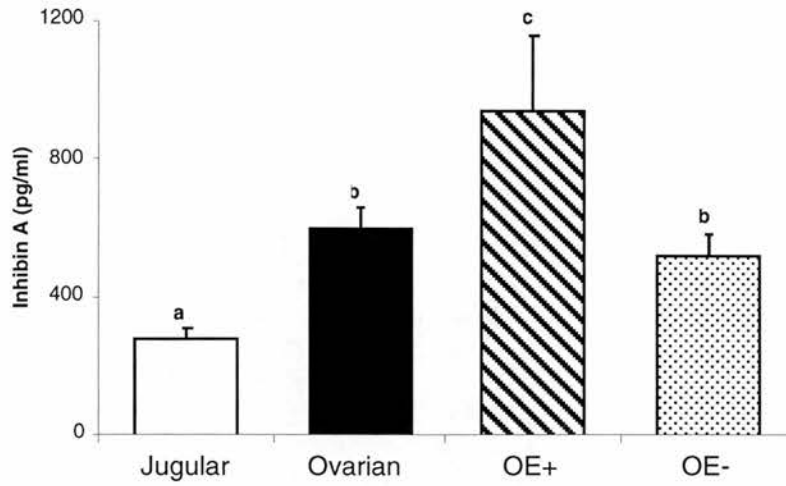


Figure 3.2 Mean \pm S.E.M. inhibin A concentrations in ovarian ($n = 36$) and jugular ($n = 18$) veins and in those veins draining an ovary containing a large oestrogenic (OE+) follicle ($n = 15$) or not ($n = 21$). Columns with different letters differ significantly ($P < 0.05$, analysis of variance).

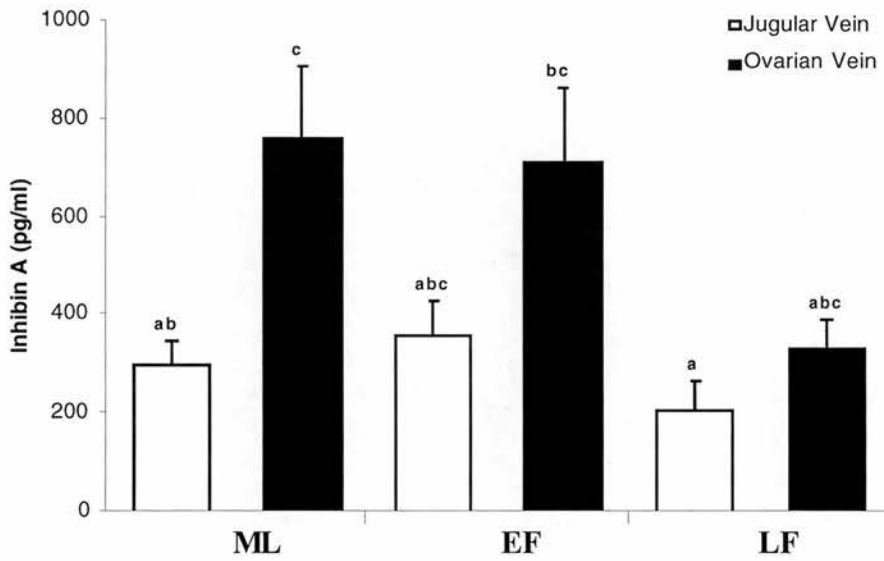


Figure 3.3 Mean \pm S.E.M. concentrations of inhibin A in jugular ($n = 18$) and ovarian veins ($n = 36$) at three stages of the oestrous cycle. Columns with different letters differ significantly ($P < 0.05$, analysis of variance).

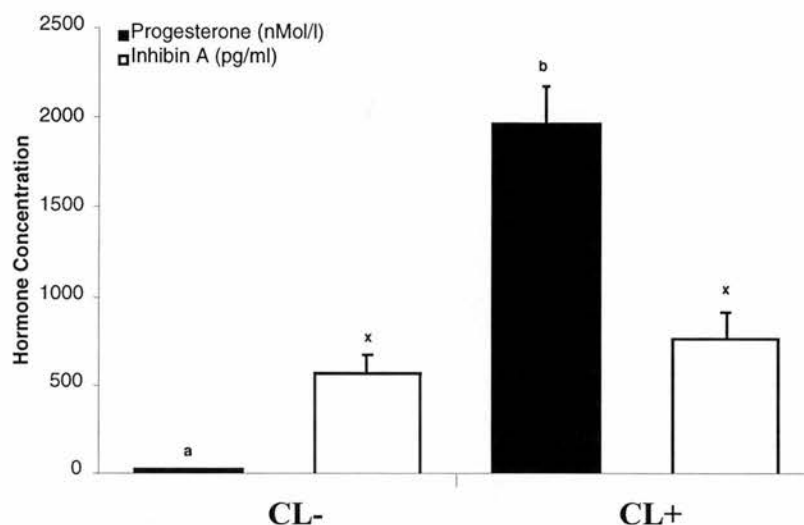


Figure 3.4 Mean \pm S.E.M. concentrations of progesterone and inhibin A in ovarian veins ($n = 36$) draining a *corpus luteum* (CL+) ($n = 27$) or not (CL-) ($n = 9$). For each hormone columns with different letters differ significantly ($P < 0.05$, analysis of variance).

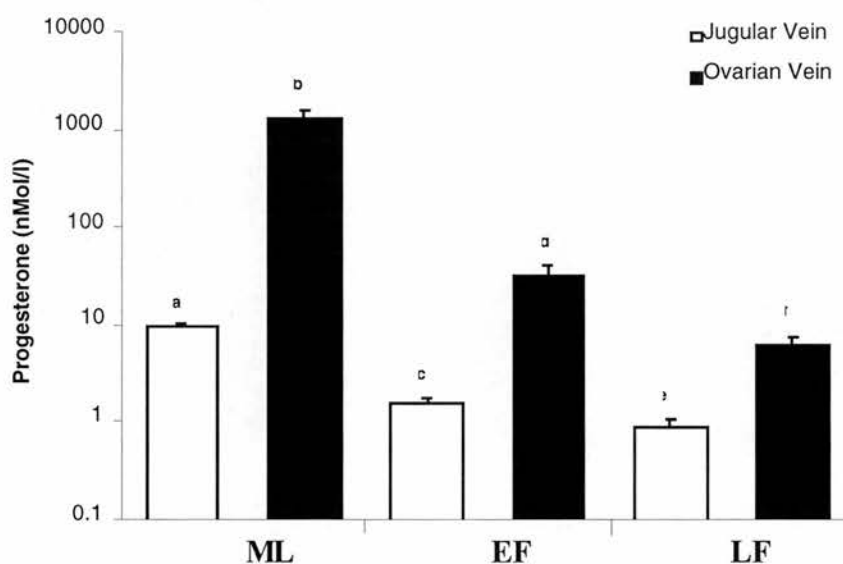


Figure 3.5 Mean \pm S.E.M. concentrations of progesterone in the jugular ($n = 18$) and ovarian vein ($n = 36$) at three stages of the oestrous cycle. Columns with different letters differ significantly ($P < 0.05$, analysis of variance).

The presence of a *corpus luteum* (CL) in an ovary, made no difference to the concentration of inhibin A recorded in the ovarian vein (Figure 3.4). In contrast, the

level of progesterone in the ovarian vein was increased 500 fold by the presence of a CL. This increase in progesterone output by an ovary containing a CL was reflected in the level of progesterone measured in the ovarian vein during the mid luteal phase, the stage of the cycle from which all CL-containing ovaries were obtained (Figure 3.5).

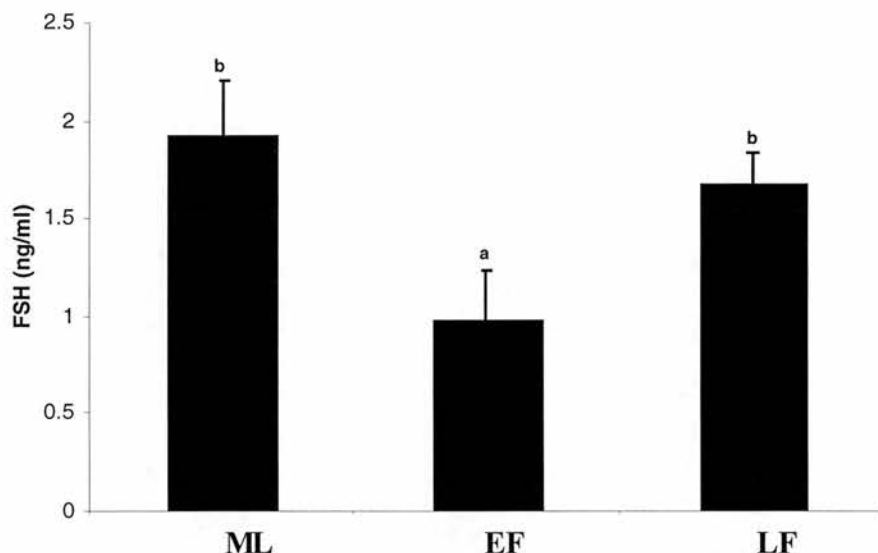


Figure 3.6 Mean \pm S.E.M. FSH concentrations in the jugular veins ($n = 18$) of ewes at three points during the oestrous cycle. Columns with different letters differ significantly ($P < 0.05$, analysis of variance).

The levels of FSH present in the circulation of the ewes studied were measured (Figure 3.6). The concentration of FSH was significantly lower during the early follicular phase of the cycle compared to that seen during the mid-luteal phase, with levels of the gonadotrophin becoming comparable with mid-luteal concentrations during the late follicular phase.

3.3.2 *In vitro* hormone measurements

Hormone production and follicle size

The follicular concentrations and release *in vitro* of inhibin A (Figure 3.7) were greater ($P < 0.001$) from large non-oestrogenic follicles ($\geq 3\text{mm}$) than from small follicles ($< 3\text{mm}$), with both measurements being greater from large oestrogenic follicles than from large non-oestrogenic follicles ($P < 0.001$). Large oestrogenic follicles, classified by parameters relating to oestradiol production, contained more

than double the amount of inhibin A in their follicular fluids compared to large non-oestrogenic follicles (757 ± 109.2 vs. 315 ± 44.4 ng/ml) and released over three times the amount of the protein (31.2 ± 8.4 vs. 8.8 ± 1.83 ng/foll/2hr).

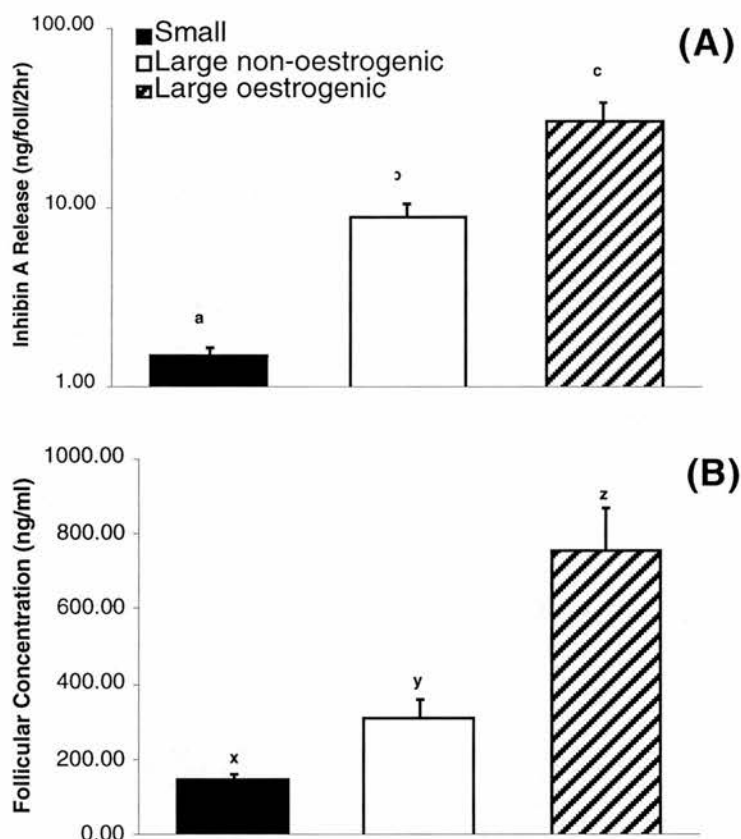


Figure 3.7 Mean \pm S.E.M. inhibin A release (A) and follicular fluid concentrations (B) from all small ($n = 488$), large non-oestrogenic ($n = 55$) and large oestrogenic ($n = 22$) follicles collected. Columns with different letters differ significantly ($P < 0.05$, analysis of variance).

During the classification of large follicles, a population of follicles (15 of 92 large follicles identified) with a follicular fluid oestradiol concentration over 100 ng/ml and an oestradiol: androstenedione ratio of less than 50 were identified. Despite their high levels of oestradiol, these follicles released very little steroid during incubation and were therefore classified as unhealthy, or atretic large follicles and were not included in either the non-oestrogenic or oestrogenic groups. Although these follicles were classified as unhealthy large follicles with respect to oestradiol production and release, the levels of inhibin A secreted by, and present in the follicular fluids of these follicles were comparable to those from large non-oestrogenic follicles with the

follicles releasing an average of 15.8 ± 4.8 ng/foll/2hr and maintaining a mean follicular fluid concentration of 475 ± 69.4 ng/ml.

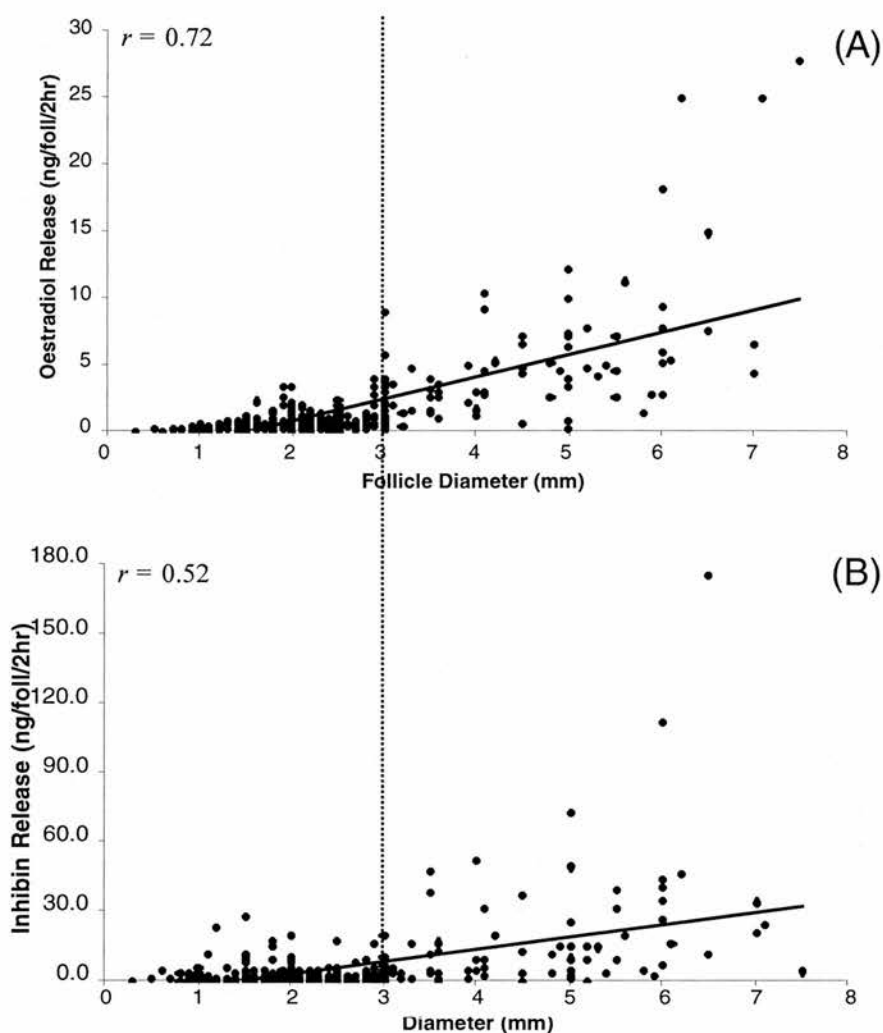


Figure 3.8 Relationship between follicle diameter and the release of (A) oestradiol ($n = 580$, $r = 0.72$, $P < 0.001$) and (B) inhibin A ($n = 580$, $r = 0.52$, $P < 0.001$, regression analysis) during 2h in vitro incubation. The dotted line represents the division between small and large follicles.

A significant correlation was found between the diameter of the follicle and the release of both oestradiol ($r = 0.72$, $P < 0.001$) and inhibin A ($r = 0.52$, $P < 0.001$) during the 2 hour incubation period (Figure 3.8).

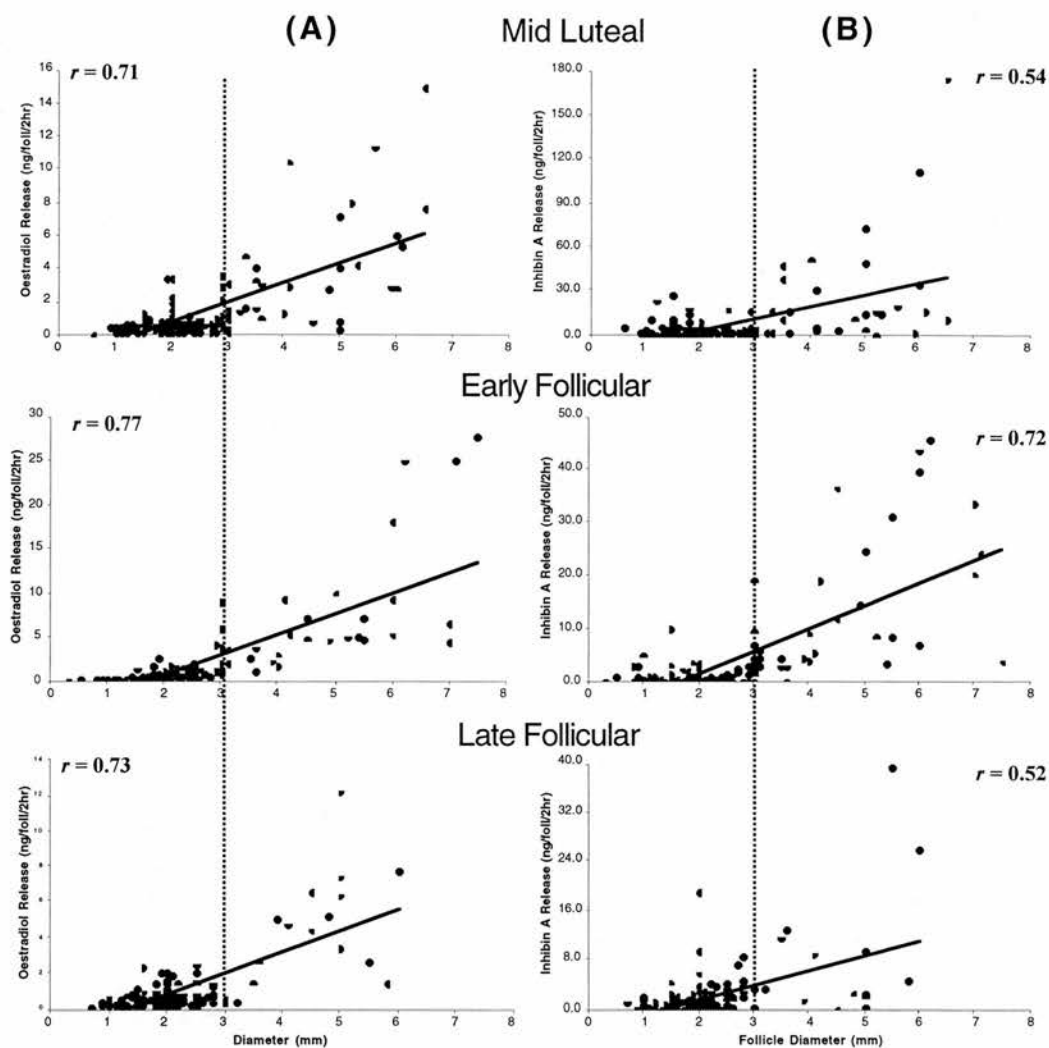


Figure 3.9 Relationship between follicle diameter and *in vitro* release of oestradiol (A) and inhibin A (B) from follicles at mid-luteal ($n = 220$), early follicular ($n = 207$) and late follicular ($n = 153$) stages of the cycle. The dotted lines represent the division between small and large follicles.

Highly significant correlations ($P < 0.001$) between follicle diameter and the release of both oestradiol and inhibin A were observed at all stages of the oestrous cycle studied (Figure 3.9). In addition, it was also noted that the correlation with inhibin A secretion was higher ($r = 0.72$) during the early follicular phase than at the other stages of the cycle.

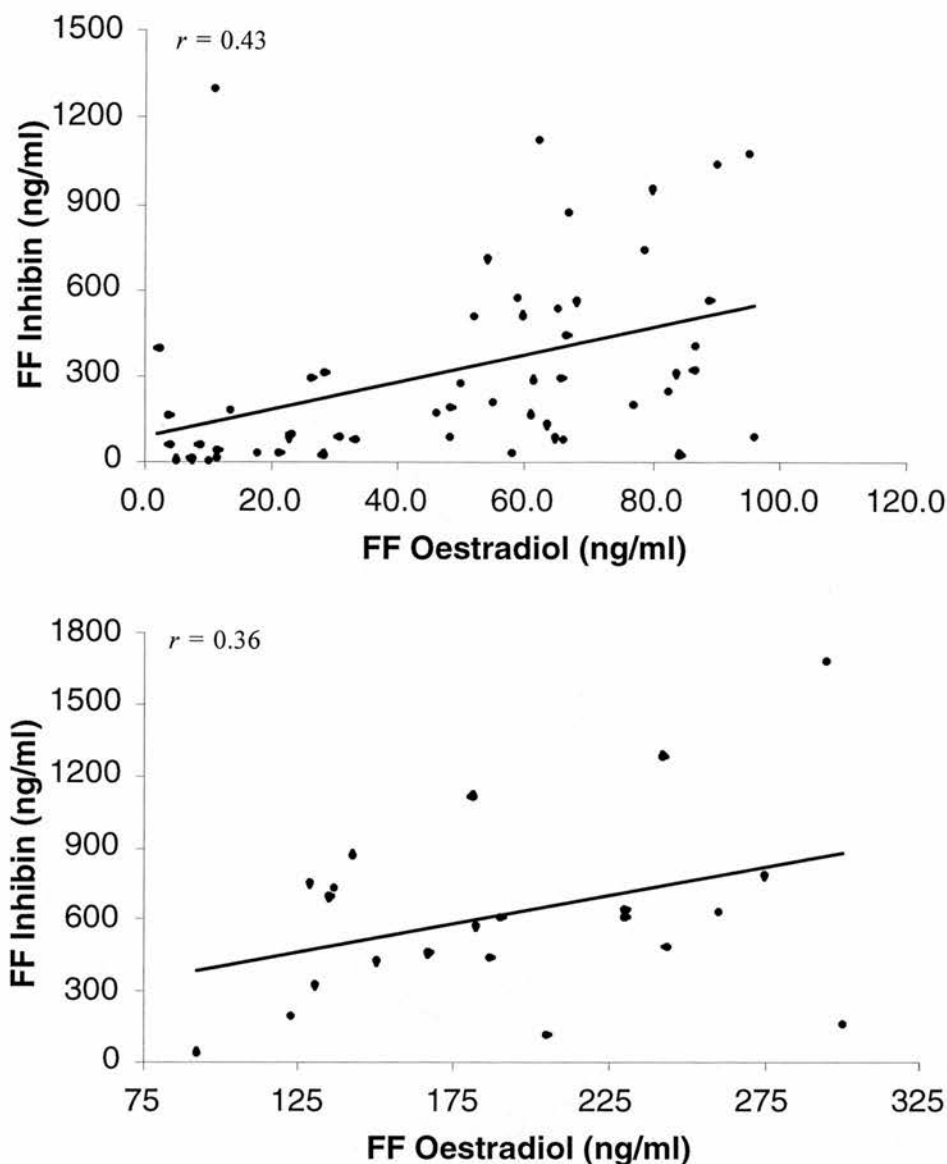


Figure 3.10 Correlation between the follicular fluid concentrations of oestradiol and inhibin A in (A) non-oestrogenic ($n = 55$, $r = 0.43$) and (B) oestrogenic follicles ($n = 22$, $r = 0.36$).

A comparison of the correlation between the follicular fluid concentrations of oestradiol and inhibin A measured in both non-oestrogenic and oestrogenic follicles showed no significant difference in correlation between the two follicle classes (Figure 3.10). Furthermore, the correlation between oestradiol:androstenedione ratio and follicular fluid inhibin A concentration did not vary significantly between non-oestrogenic, oestrogenic and large unhealthy follicles (Figure 3.11).

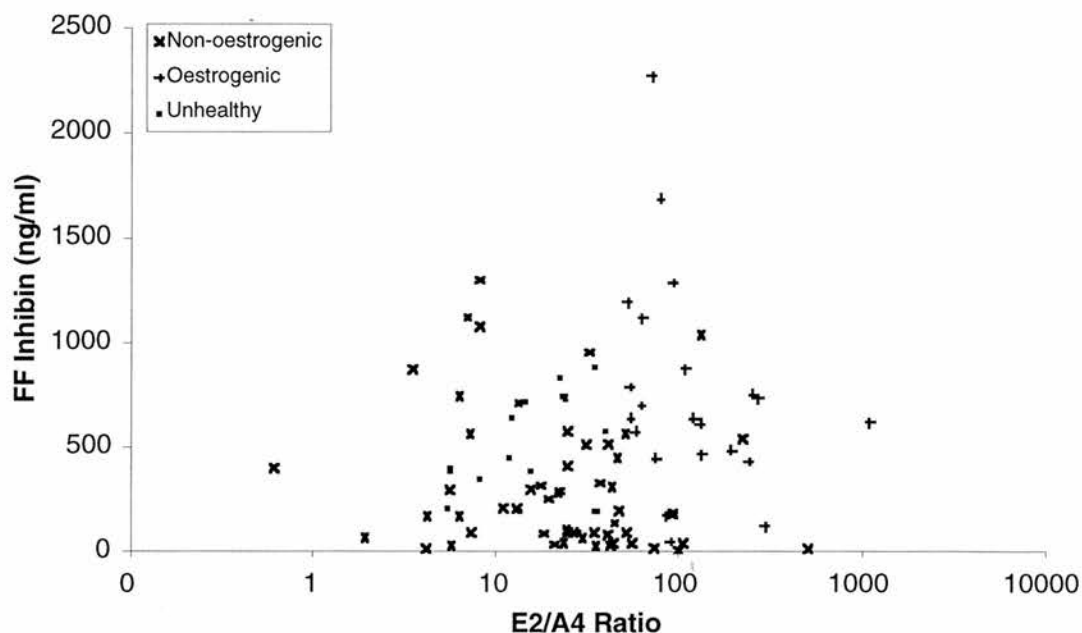


Figure 3.11 Relationship between the oestradiol:androstenedione ratio and inhibin concentration in the follicular fluids of large oestrogenic ($n = 22$), non-oestrogenic ($n = 55$) and follicles classified as unhealthy ($n = 15$).

Hormone production and stage of the oestrous cycle

Large follicles collected during the early follicular phase released more ($P = <0.001$) oestradiol than large follicles dissected from ovaries at the other stages. In contrast inhibin A secretion by large follicles was comparable during the luteal and early follicular phases but declined significantly ($P < 0.001$) during the late follicular phase (Figure 3.12A). The concentration of oestradiol in the follicular fluid of large follicles after a 2 hour incubation reflected the change in release of the steroid with a significant fall ($P < 0.001$) in the late follicular phase. In contrast there was no change in the concentration of inhibin A or androstenedione in the follicular fluid of large follicles throughout the cycle (Figure 3.12B).

There was little change in the concentration of steroids or inhibin A in the follicular fluid of small follicles during the cycle or in their release during incubation. There was, however, a significant increase ($P < 0.001$) in the mean concentration of oestradiol in the follicular fluid of small follicles collected during the follicular phase

compared to those from the luteal phase, although this rise was not reflected in the level of the steroid released *in vitro*. The release of inhibin A by small follicles peaked ($P < 0.001$) during the mid luteal phase, however, there was no change in the concentration of the hormone in the follicular fluid of these follicles.

Large oestrogenic follicles maintained higher concentrations of inhibin A in their follicular fluid and released significantly more ($P < 0.001$) of the dimer than either small follicles or large non-oestrogenic follicles during the luteal and early follicular phase (Figure 3.13). Although the concentration of inhibin A in follicular fluid of large oestrogenic follicles was comparable at all stages of the cycle, large oestrogenic follicles collected during the mid luteal phase released significantly more ($P < 0.001$) inhibin A than those from follicular phase ovaries. The concentration of inhibin A in the follicular fluid and the level of release of the hormone from small follicles and large non-oestrogenic follicles did not vary between the stages of the cycle. The concentration of inhibin A in the follicular fluid of large non-oestrogenic follicles was significantly higher than the level measured in small follicles during the early follicular phase, however, the release of inhibin A by large non-oestrogenic follicles was only greater than that of small follicles during the luteal phase.

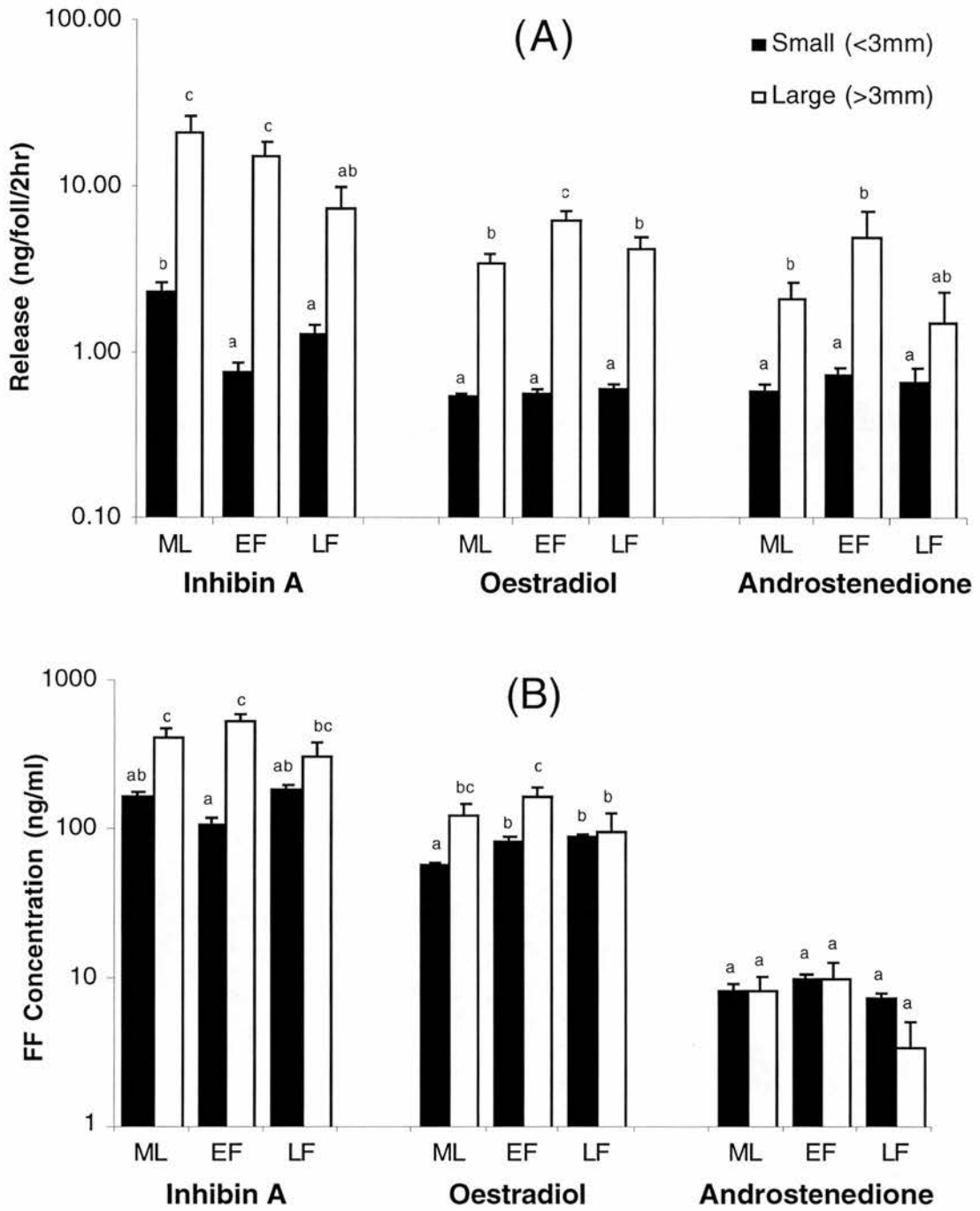


Figure 3.12 Mean \pm S.E.M. release (A) and follicular fluid concentrations (B) of inhibin A, oestradiol and androstenedione after 2h incubation *in vitro* from small ($n = 488$) and large follicles ($n = 92$) at three stages of the oestrous cycle. For each hormone columns with different letters differ significantly ($P < 0.05$, analysis of variance).

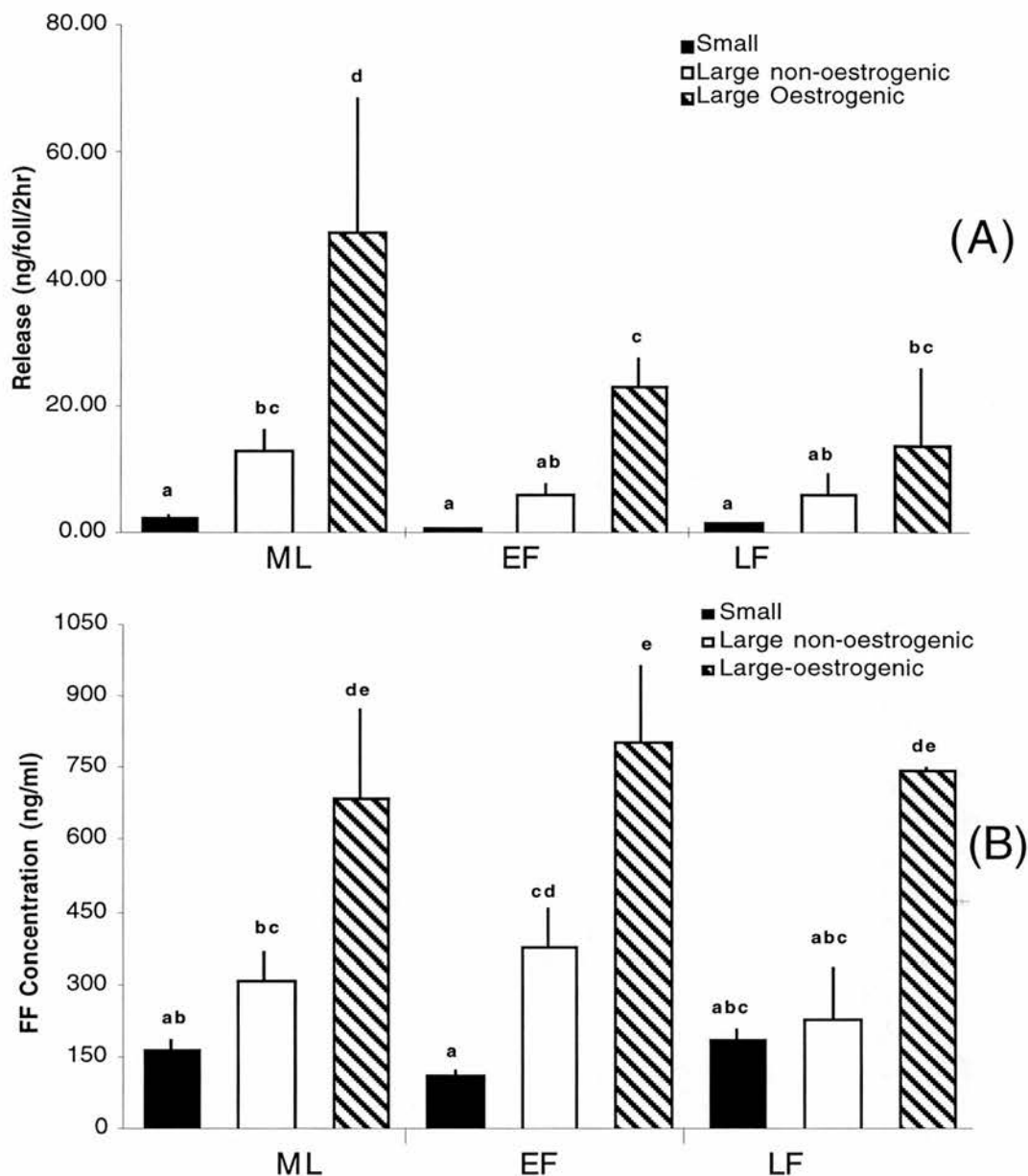


Figure 3.13 Mean \pm S.E.M. inhibin A release (A) and follicular fluid concentration (B) from small, large non-oestrogenic and large oestrogenic follicles from three stages of the oestrous cycle. Columns with different letters differ significantly ($P < 0.05$, analysis of variance).

3.4 Discussion

Our study has confirmed that the ovary secretes a large amount of inhibin A at all stages of the oestrous cycle of the ewe. The fact that the concentration of inhibin A is highest in the blood draining an ovary containing a large oestrogenic follicle (Figure 3.2) strongly suggests that this follicular population is the major source of

inhibin A secreted by the ovary. Additionally, the fact that levels of inhibin A measured in ovarian veins draining ovaries without large oestrogenic follicles were consistently greater than the jugular levels suggests that other structures within the ovary secrete the hormone in addition to preovulatory follicles. The observation that the presence of a *corpus luteum* makes little difference to the concentration of inhibin A measured in the ovarian vein (Figure 3.4) is consistent with the previous suggestion that the ovine CL does not produce inhibin (Mann, *et al.*, 1989). However, as widely noted in the literature (Baird, *et al.*, 1973), the CL is a major source of progesterone in the ovine ovary during the luteal phase of the cycle (Figure 3.5).

The concentration of inhibin A in follicular fluid is extremely high in large oestrogenic follicles compared to large non-oestrogenic and small follicles. Large oestrogenic follicles secrete greater amounts *in vitro* than follicles from the other two classes (Figure 3.7). Although large non-oestrogenic and small follicles do not secrete as much inhibin A as large oestrogenic follicles, they still contribute significantly to the overall production of the hormone by the ovary. The concentration of inhibin A in ovarian venous blood draining an ovary containing only large non-oestrogenic follicles and small follicles is higher than that measured in jugular venous blood (Figure 3.2).

The overall production of inhibin A *in vitro* is greater from large non-oestrogenic than from small follicles. However, the fact that there are so many more small follicles than large follicles makes it difficult to assess the relative contribution of the two classes to the total secretion of inhibin A by the ovary.

Although there are large oestrogenic follicles present in the ovaries at each stage of the cycle studied, oestradiol secretion by large follicles peaks during the early follicular phase. The rise in oestradiol secretion *in vivo* is dependant on the stimulation of the follicle by the marked increase in LH pulse frequency which occurs during the luteal-follicular transition (Baird, 1978b). Each pulse of LH stimulates an increase in the production of androstenedione by the theca cells of the follicle (Campbell, *et al.*, 1998a) which is then used by the granulosa compartment of the pre-ovulatory follicle to produce oestradiol (McNeilly, *et al.*, 1984). The significant increase in oestradiol release, *in vitro*, by large follicles dissected during the early

follicular phase probably reflects the increase in *in vivo* gonadotrophin stimulation prior to follicle collection.

There was a significant fall in the concentration of oestradiol in the follicular fluid and in release *in vitro* from large follicles collected in the late follicular phase (Figure 3.12). These ovaries were collected 24 hours after the onset of oestrus, within a few hours of ovulation. Therefore it was assumed that these ovaries had been exposed to the preovulatory LH surge, which occurs within 4-8 hours of the onset of oestrus (Cumming, *et al.*, 1973). This fall in oestradiol concentration is consistent with the marked decline in oestradiol secretion observed at this stage of the cycle (Baird and McNeilly, 1981).

The surge levels of LH induce profound changes in the structure and function of the pre-ovulatory follicle bringing about the initial stimulation and subsequent inhibition of both androstenedione and oestradiol secretion (Baird and McNeilly, 1981). Luteinisation of the granulosa cells is initiated in anticipation of production of progesterone by the *corpus luteum*. The significant decline in the follicular fluid concentration and release *in vitro* of oestradiol from large follicles collected during the late follicular phase reflects this inhibition of steroid synthesis.

The follicular fluid concentration and release *in vitro* of inhibin A was lower in large follicles collected during the late follicular phase compared to those dissected earlier in the same phase or in the luteal phase (Figure 3.12). This decrease in the release of inhibin A is likely to reflect a loss of phenotype in large oestrogenic follicles associated with the luteinisation of both their granulosa cells due to the preovulatory surge of LH. This hypothesis is supported by the fact that luteal tissue does not secrete either oestradiol or inhibin A in the ewe (Mann, *et al.*, 1989) and also that peripheral inhibin A (Knight, *et al.*, 1998) and inhibin secretion (Souza, *et al.*, 1997b) fall during the follicular phase.

In addition to increasing steroid output by the theca and granulosa cells, the LH surge also affects the ability of granulosa cells to produce inhibin A. Within 24 hours of the LH surge the expression of mRNA for the inhibin α and β A subunits in granulosa-lutein cells of the peri-ovulatory follicle is inhibited (Engelhardt, *et al.*, 1993), although β B subunit mRNA expression persists in the mature corpus luteum.

The exact timing of these changes is not known but it may explain the significant fall in the secretion of inhibin A in the ewe immediately after ovulation.

Additionally, the level of inhibin A released by small antral follicles was lower in follicular phase ovaries than from those removed during the luteal phase (Figure 3.13). This decline may reflect the high levels of atresia observed in subordinate follicles during the follicular phase which is essential for the selection of a specified number of ovulatory follicles.

The maximal secretion of inhibin A from large oestrogenic follicles during the luteal phase may be due to the fact that inhibin A production, unlike oestradiol, is not dependant on LH, but rather FSH. The synthesis of inhibin A by the granulosa cells is stimulated by FSH (Campbell and Baird, 2001), the levels of which fall progressively throughout the follicular phase reaching a nadir just prior to ovulation.

Therefore, the reduction in inhibin A production by large oestrogenic follicles in the follicular phase may be attributable to the low levels of FSH released by the pituitary at this stage of the cycle (Figure 3.6). However, the fall in inhibin A release during the early follicular phase was not reflected in the peripheral levels of the hormone measured. Therefore this decrease in output of the dimer by small and large oestrogenic follicles may be due to the *in vitro* environment and it is possible that follicles from the EF phase are more susceptible to the removal of gonadotrophins and local factors present *in vivo*. It may also be possible that there is a structural difference between the follicles at the two stages, which has little effect *in vivo*, with those follicles during the ML stage being more permeable to inhibin A.

In conclusion, we have shown that the main source of ovarian inhibin A in the sheep are large oestrogenic follicles with a significant quantity of the hormone produced and released by both small antral and large non-oestrogenic follicles. In addition, we note a decrease in the secretion of the dimer by small antral and large oestrogenic follicles during the early follicular phase which may be attributable to the recorded fall in FSH in the periphery at this time. It should be noted that at this point during the follicular phase, selection of ovulatory follicles has already occurred and therefore the fall in inhibin A secretion is likely to reflect the large number of follicles which are in the early stages of atresia.

Chapter 4 – Expression of inhibin subunits throughout the oestrous cycle of the ewe

4.1 Introduction

The pituitary-derived gonadotrophins FSH and LH together form the primary trophic stimulus for follicle growth and development in mammals. In addition to FSH and LH, the influences of a cohort of numerous local ovarian factors in the control of the process have also been reported. The granulosa cell-derived ovarian peptides inhibin and activin have considerable effects on follicle development at the level of the pituitary with inhibin acting to inhibit FSH release (Ling, *et al.*, 1985) and locally produced activin directly opposing this effect (Carroll, *et al.*, 1991b).

As well as their modulation of FSH release from the brain, inhibin and activin are able to affect the growth and differentiation of many cell types and their subunits have been recorded in numerous tissues throughout the mammalian body, including the kidneys (Kojima *et al.*, 2001), liver (Lau *et al.*, 2000), prostate (Mellor *et al.*, 2000), adrenal glands (Munro *et al.*, 1999) and the heart (Ladd *et al.*, 1998). Activins are synthesized at high levels in cancerous tissues, including cancer of the prostate (Dowling and Risbridger, 2000), ovarian epithelial tumours (Zheng *et al.*, 2000) and are implicated in the growth of these tumours. Activin has also been implicated as an essential molecule in the modulation of tissue repair, with high-level expression at sites of trauma (Hubner *et al.*, 1999) and may in addition contribute to rheumatoid arthritis (Gribi *et al.*, 2001).

Inhibins on the other hand do not seem to have such a wide range of physiological effects with the majority of their reported effects taking place in the brain and gonads.

Studies examining the expression of inhibin subunits in the mammalian ovary have revealed differential synthesis of the subunits depending on the stage of follicle development. Early work concerned with the expression of inhibin subunit mRNAs throughout the oestrous cycle of the ewe (Rodgers *et al.*, 1989; Tisdall, *et al.*, 1994) detected mRNA signal for both the α and β A subunits in only the granulosa cells of healthy antral follicles with no changes in expression throughout the cycle. Another

report detected β B subunit mRNA in only the granulosa cells of healthy antral follicles (Engelhardt, *et al.*, 1993) along with mRNA for both the α and β A subunits, however, in contrast to the other research discussed, inhibition of inhibin mRNA expression was found shortly after the LH surge, concomitant with the fall in oestradiol release by the ovarian follicles.

In each of these studies no evidence of mRNA for either subunit in ovine *corpora lutea* is presented, which is consistent with previous findings suggesting that the *corpus luteum* of the ewe does not secrete inhibin (Mann, *et al.*, 1989) despite earlier reports to the contrary (Tsonis *et al.*, 1988). In contrast to the sheep, human and primate *corpora lutea* express both α and β A subunit mRNA and protein in their luteinised granulosa cells (Schwall *et al.*, 1990; Smith *et al.*, 1991; Roberts, *et al.*, 1993) and secrete bioactive inhibin A (Davis *et al.*, 1987).

More recently, research concerned with the ovine ovary, measured protein for both β A and β B subunits in the granulosa cells of follicles of all sizes, including primordial follicles. Interestingly, mRNA for the β -subunits was only evident in specific follicle types (McNatty, *et al.*, 2000). Expression of mRNA for both α and β B subunits was observed in small preantral follicles (2-4 layers of granulosa cells), larger preantral follicles and antral follicles, with β A subunit mRNA confined to the granulosa cells of antral follicles. Detection of α subunit protein correlated exactly with mRNA localisation, with the subunit measured in the granulosa cells of small preantral, large preantral and antral follicles. In contrast, both β subunit proteins were detected in all follicle types, including primordial and primary follicles, whereas mRNA for these proteins was not detected. These observations suggest that the β subunit protein present in primordial and primary follicles is not synthesized by the follicles themselves and has an origin in another ovarian compartment.

If inhibins and activins are involved in the growth and development of ovarian follicles, variation in the expression of their subunits may be expected throughout the ovine oestrous cycle. Previous work has examined the expression of mRNA for the inhibin subunits throughout the cycle but not the proteins for which they code. The aim of this study was to detect both inhibin and activin subunit mRNA and protein

message in ovaries removed from sheep at three time points during the 17 day oestrous cycle.

The research described in Chapter 3 utilised a specific assay for inhibin A to measure levels of the hormone secreted by antral follicles, however, inhibin B is not detectable in ovine plasma or follicular fluid using a specific assay (McNeilly, 2000), indicating that the dimer is unlikely to be produced in the ewe. Therefore this work was carried out in order to identify any follicle populations co-expressing both α and β B subunit protein within their granulosa cells, suggesting the possible presence of inhibin B.

In the course of this study diffuse staining for inhibin β subunits was noted in the theca layers of follicles, therefore immunostaining for the related factor anti Mullerian hormone (AMH) was undertaken as a validation to confirm whether this staining was artefactual.

4.2 Materials and Methods

Materials

4.2.1 Animal Tissue

Ovaries sectioned for immunochemical detection and *in situ* hybridisation were obtained from 6 Scottish Blackface ewes during the breeding season throughout the period October to December 1998 at the Marshall Building, University of Edinburgh, Lothian.

Oestrous cycles were synchronised using medroxyprogesterone acetate sponges (60 mg/sponge, Upjohn Animal Health Division, Crawley, Sussex, UK) followed by i.m. injection of 100mg cloprostenol, a potent prostaglandin $F_{2\alpha}$ analogue (Estrumate, Cooper's Animal Health Ltd, Crewe, Cheshire, UK). The synchronised ewes were then allocated to one of three groups of two animals and underwent surgery at one of three time points during the oestrous cycle: 24 hours after oestrus (day 2), mid-luteal (day 10), and early in the follicular phase (day 15). Time points for ovariectomy were selected to provide ovaries with different follicular profiles.

4.2.2 Primary antibodies

For detection of the α -subunit, a rabbit polyclonal antibody (R150) raised against amino acids 1-26 of the N-terminus of the porcine inhibin α -subunit conjugated to ovalbumin (Professor A. S. McNeilly, HRSU, Edinburgh, UK) was employed. Mouse monoclonal antibodies raised against amino acids 82-114 of the β A subunit of human inhibin (E4 clone) and amino acids 82-114 of the human β B subunit (12/13 clone), were supplied by Professor Nigel Groome, Oxford Brookes University, UK. The polyclonal anti-Mullerian hormone (AMH) antibody raised in the rabbit was supplied by Dr Nathalie Josso (INSERM U493, France).

Methods

4.2.3 Ovariectomy

Anaesthesia was induced by minimal quantities (200-300 mg) of a mixture of two parts thiopentone (Intraval; RMB Animal Health Ltd, Dagenham, Essex, UK) and one part pentobarbitone sodium (Sagatal; May and Baker Ltd, Dagenham, Essex, UK) and maintained using halothane (1.5 – 2.5%). Mid-ventral laparotomy was then performed and the reproductive tract exteriorised. The ovaries were removed and fixed overnight in 4% (w/v) paraformaldehyde in PBS before being transferred to 70% ethanol.

4.2.4 Immunohistochemistry

Ovaries were halved using a scalpel, processed for histology and embedded in paraffin wax blocks. 5 μ m sections were then cut, the wax removed from the sections using xylene and rehydrated in an ethanol gradient. The sections were incubated in a 70% methanol: 30% hydrogen peroxide solution for 30 minutes (to block any endogenous peroxidase activity) prior to a 1 hour incubation with a 20% non-specific serum in PBS blocking solution. Sections were then incubated with primary antibodies, targeted against the inhibin subunits, for one hour, washed with PBS and incubated for a further hour with the corresponding fluorescent second antibody (Sigma). The slides were mounted with 50 μ l Citifluor (Citifluor Ltd, London) and the

staining visualised using a Zeiss 510 Laser scanning confocal microscope (Carl Zeiss Microscopy, Jena, Germany).

4.2.5 *In situ* Hybridisation

Pre-hybridisation

³⁵S labelled sense and antisense riboprobes were produced from purified pGEMTeasy plasmids (Promega, UK) containing inhibin subunit cDNAs kindly provided by Dr C. MacDougall, ICMB, Edinburgh, UK. Plasmids were cleaved using specific restriction enzymes to provide inserts (Table 4.1) which were then labelled (see appendix) using either T3 or T7 polymerases. Specific activities were calculated and the level of incorporation for each probe was measured to be at least 10%

Construct	Insert	Restriction Enzyme	Polymerase
pGC1	α - Sense	SpeI	T7
pGC2	α - Antisense	SpeI	T7
pGC3	β A - Sense	XbaI	T7
pGC4	β A - Antisense	XbaI	T7
pIN β B	β B - Sense	BamHI	T7
pIN β B	β B - Antisense	SphI	T3

Table 4.1 Restriction enzymes used for the preparation of sense and antisense inhibin mRNA probes.

Ovarian tissue was fixed and sectioned as previously described (Chapter 2). Tissue sections were rehydrated using an alcohol gradient, postfixed in 4% paraformaldehyde (PFA) in PBS for 20 minutes, washed with PBS and then digested with proteinase K (2 μ g/ml) in a 0.1M Tris-HCl, 100mM EDTA solution at 37°C. The sections were then re-fixed in 4% PFA, immersed in 2 X SSC and then washed in 0.25% acetic anhydride in 0.1M triethanolamine-HCl. Following a further treatment with 2 X SSC, the slides were dehydrated in a series of alcohols and allowed to dry in a fume cupboard.

Hybridisation

Probes were diluted in hybridisation buffer to 1×10^4 c.p.m/ μ l. 50 μ l of the probe was added to each slide and hybridisation performed in a sealed container, humidified with 50% formamide in 2 X SSC, for 20 hours at 55°C

Post-hybridisation

Slides were washed twice with 2 X SSC at 55°C prior to incubation with RNase A (20 μ g/ml in 2 X SSC) at 37°C for 1 hour. The slides were then treated with a series of solutions containing decreasing salt concentrations and varying quantities of β -mercaptoethanol and formamide (See Appendix for full details) before being dehydrated as before. The slides were then dried at room temperature, dipped in liquid emulsion (Kodak K2; Kodak) at 42°C, transferred to a light proof box and exposed for three weeks. Slides were developed in a dark room for 2.5 minutes in Kodak D-19 developer, washed for 30 seconds in water and then fixed with Hypam fixer (ILFORD Ltd., Cheshire, UK) for 3 minutes. The developed tissue sections were then counter-stained with haemotoxylin, dehydrated, mounted and the hybridisation visualised under bright and dark field condensers using a Nikon Elipse E800 microscope (Nikon UK Ltd).

4.2.6 Measurement of staining intensity

Levels of immunostaining in the experimental tissue sections were quantified using Scion Image graphical analysis software (Scion Corp, USA). The average pixel intensity (on a scale from black to white, with black as 0 and white as 100) within a rectangular area was measured for each cellular compartment. Four rectangles of a consistent size were analysed from each compartment of each individual follicle, with a minimum of five follicles per size class. Levels of staining measured in the adjacent stromal tissue were then subtracted from all follicular cell values as a control. Mean values of pixel intensity for each compartment of each follicle size were then calculated and plotted. The mean number of follicles examined for each size class at each stage of the cycle was 6.8 ± 0.29 follicles/class. This method of measuring

staining intensity was validated (Chapter 2 – Section 2.8.3) to ensure minimal variation between different slides included in the same run of experiments.

4.2.7 Follicle Classification

Follicles were classified into one of six groups using a number of criteria previously described (McNatty, *et al.*, 2000). This system of classification focuses on both the follicle size and the extent of the growth and proliferation of the granulosa cells of the follicles. Each follicle studied under the microscope was measured using image analysis software and classified using the criteria listed in Table 4.2.

Follicle Type	Primary	Small Preantral	Large Preantral	Early Antral	Medium Antral	Large Antral
Layers of Granulosa cells	1-<2	2-<4	4-<6	>5	>5	>5
Follicle Diameter (µm)	50-100	100-200	200-300	300-<2000	2000-4000	>4000

Table 4.2 Criteria used for follicle classification on immunofluorescent slides.

4.3 Results

4.3.1 Immunohistochemistry

Staining for both β -subunits was evident in the granulosa cells of follicles at all stages of development, with α -subunit only detectable in the granulosa compartment of large preantral and antral follicles (Figure 4.1). However, this pattern was not evident in all follicles. A small number of apparently healthy follicles, at both preantral and antral stages of development showed no staining for any subunit, or very much reduced levels compared to those detected in other follicles. These follicles were not specific to one time point and were noted throughout the cycle.

There were no significant differences in the levels of expression of inhibin subunits at the three stages of the cycle (Summarised in Table 4.3), with the patterns of expression comparable with those illustrated in Figure 4.1 in each case.

	Stage of Follicle	Stage of Oestrous Cycle		
		ML	EF	LF
α Subunit	Pri	-	-	-
	SP	-	-	-
	LP	*	*	*
	SA	*	*	*
	MA	* *	* *	* *
	LA	* *	* *	* *
βA Subunit	Pri	*	*	*
	SP	*	*	*
	LP	*	*	*
	SA	*	*	*
	MA	* *	* *	* *
	LA	* *	* *	* *
βB Subunit	Pri	*	*	*
	SP	*	*	*
	LP	* *	* *	* *
	SA	* *	* *	* *
	MA	* *	* *	* *
	LA	* *	* *	* *

Table 4.3 Inhibin subunit staining in follicles at increasing stages of development at different stages of the oestrous cycle. Pri – Primary; SP – Small preantral; LP – Large Preantral; SA – Small antral; MA – Medium antral; LA – Large antral. Positive staining is indicated by * and strong staining by **.

Detection of α -subunit protein was found primarily in the granulosa cells, with low level staining measured in the theca interna of some antral follicles. Diffuse immunostaining for both β -subunits was also evident in the theca interna (TI) and theca externa (TE) compartments of some large preantral follicles and a large number of healthy antral follicles.

The presence of βA subunit protein in the theca externa was seen in follicles from large preantral to medium antral size, with no measurable level in large antral follicles, particularly in those follicles expressing a high level of α -subunit.

Profiles of the levels of α and βB immunostaining across the compartments of a follicle (Figure 4.2) indicate a high level of staining for βB in the theca layers relative to the peak levels seen in the granulosa, whereas the level of α staining in the theca is low compared to the granulosa.

Staining for β -subunits in the thecal compartments of follicles (where present) was confirmed by quantification, (Figure 4.3) which indicated a level of staining in the theca cells higher than that of the stromal tissue surrounding the follicles.

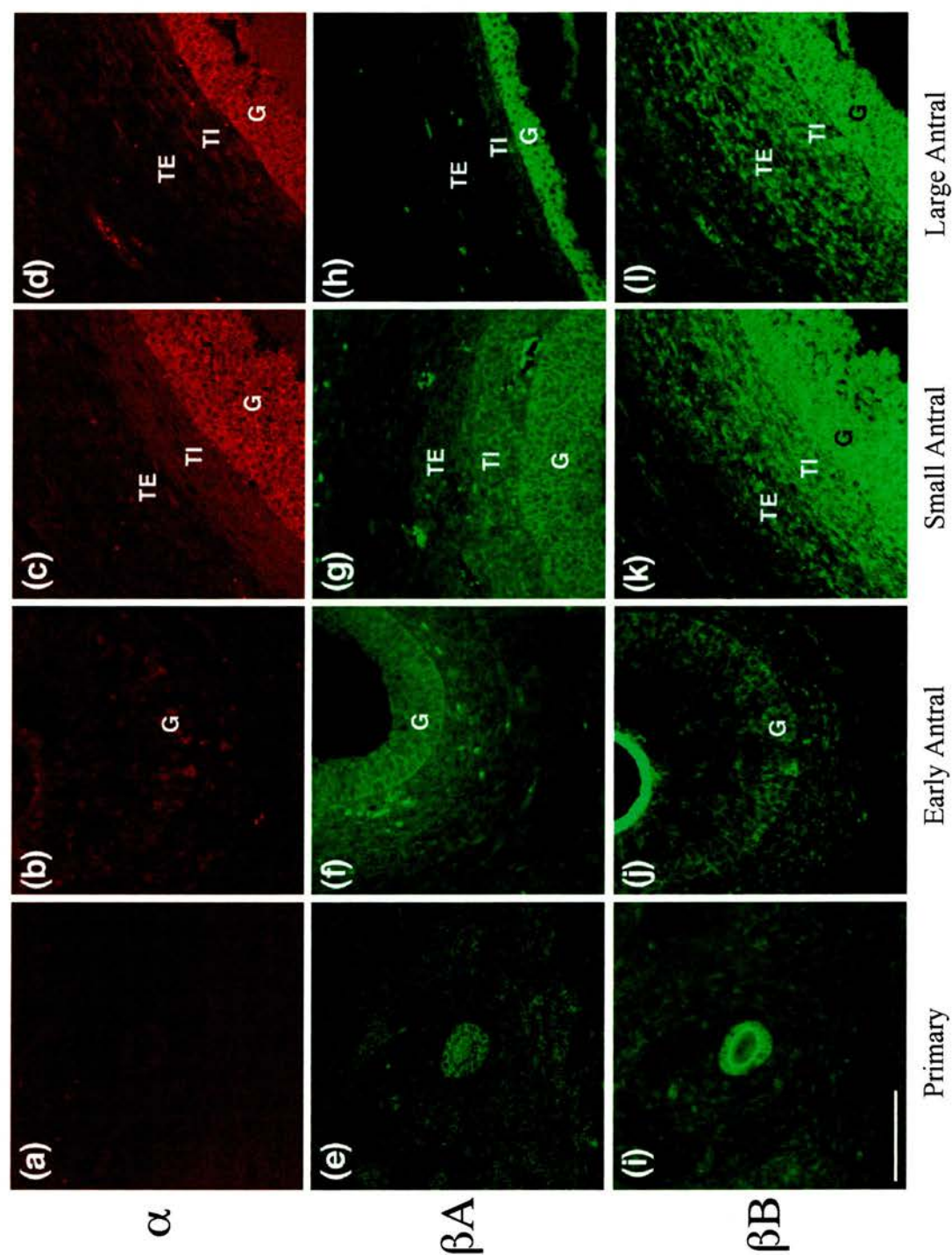


Figure 4.1 Immunostaining for inhibin alpha subunit, β A subunit and β B subunit protein in follicles at increasing levels of development. G – granulosa; TI – theca interna; TE – theca externa. All images are x400 magnification. Scale Bar represents 100 μ m.

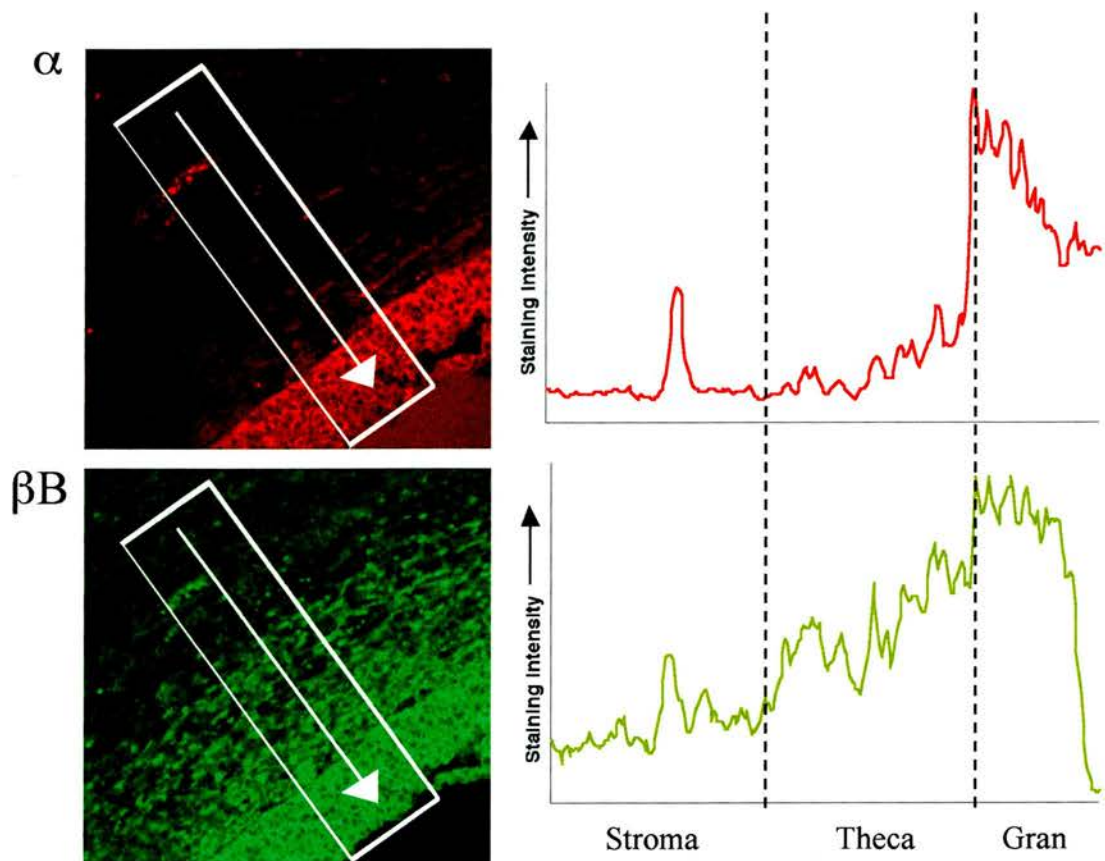


Figure 4.2 Immunostaining profile across the different compartments of a large antral follicle incubated with antibodies raised against both the α and β B inhibin subunits.

The measurement of inhibin A secretion from follicles at different stages of development (Chapter 3) has shown that healthy antral follicles are capable of production of this particular dimer. Dual fluorescent labelling of ovary sections with antibodies raised against both the α and β B subunit of inhibin (Figure 4.4), revealed the expression of both proteins in the granulosa cells of antral follicles, again with a diffuse pattern of β B staining observed in the theca layers in these follicles as well as late preantral follicles.

In addition to the β subunit staining observed in the theca layers, a ring of very intense binding of the primary antibody raised against the β B subunit was noted in the area immediately surrounding the oocyte (zona pellucida) in follicles at all stages of development (Figures 4.1 and 4.4).

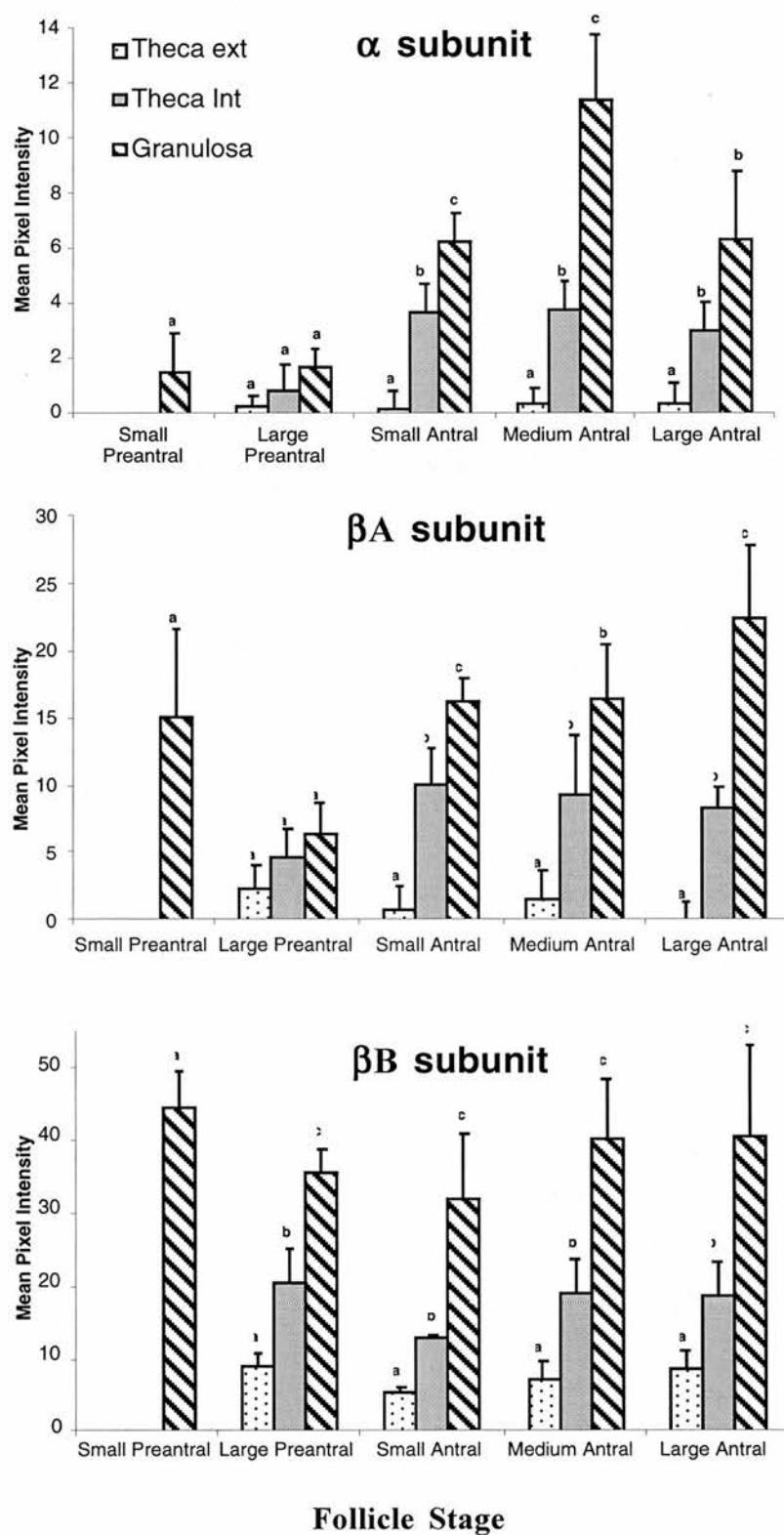


Figure 4.3 Inhibin α , βA and βB subunit quantification at increasing levels of follicle development. $n = 30$ for each class of follicle. For each follicle group, columns with different letters differ significantly ($P < 0.05$, analysis of variance).

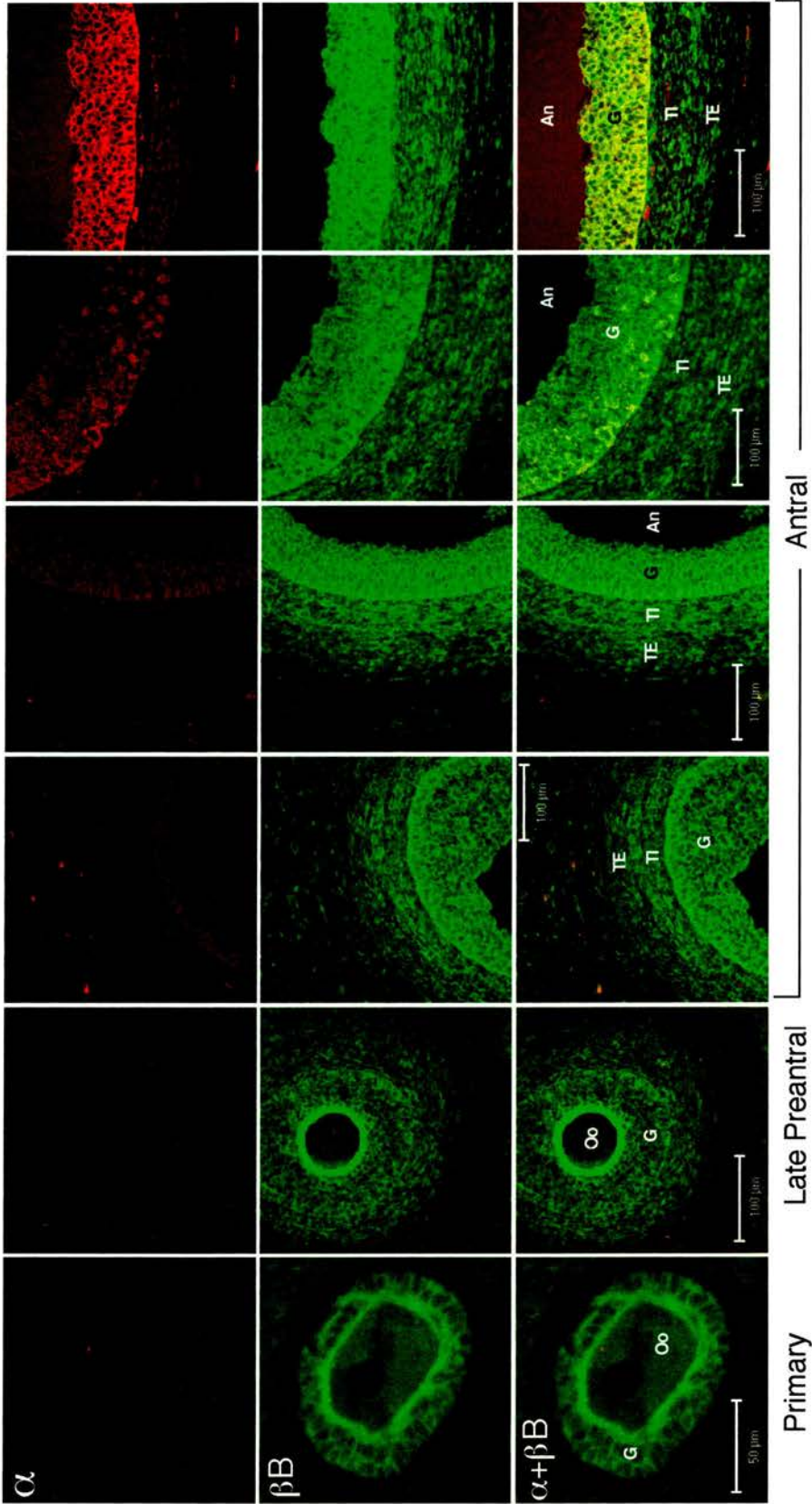


Figure 4.4 Single and Dual fluorescent labelling for inhibin α and β B subunit protein in follicles at increasing levels of development. Yellow staining represents colocalisation of both α and β B subunit proteins. G – granulosa; TI – theca interna; TE – theca externa; Oo – Oocyte; An – Antrum.

Examination of inhibin subunit protein expression in the ovine *corpus luteum* revealed the presence of β B subunit-containing factors (Figure 4.5). No staining was observed in luteal tissue incubated with antibodies raised against either the α or β A subunits.

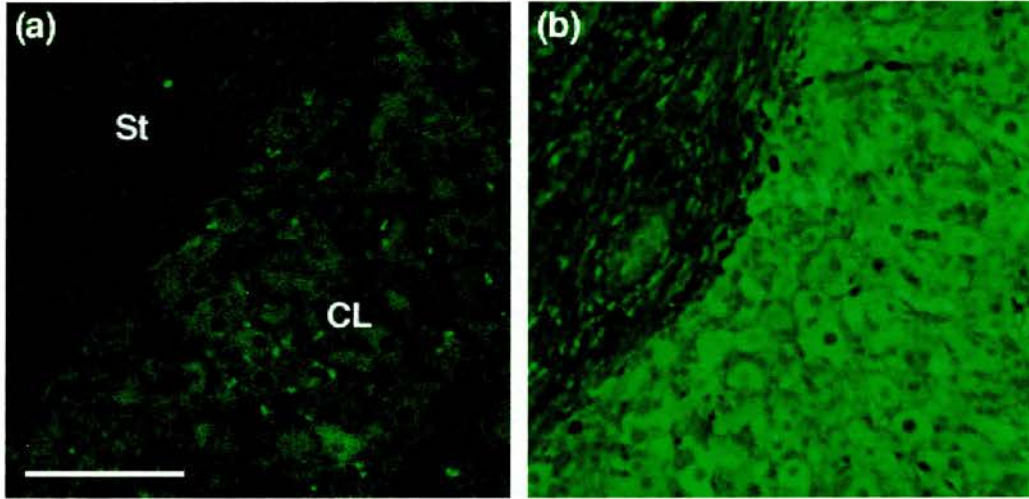


Figure 4.5 Immunostaining for β B subunit (b) in a mid-luteal (day 10) *corpus luteum* compared with control staining (a). x400 magnification. Scale Bar represents 100 μ m. St – Stroma; CL – Luteal tissue.

4.3.2 *In situ* Hybridisation

In situ hybridisation only detected inhibin subunit mRNAs in granulosa cells, with no staining visible in any other ovarian compartment including the theca cells. There was no hybridisation with any of the three probes in primordial or primary follicles at any of the time points during the cycle, with large antral follicles expressed high levels of all three subunits (Figure 4.6).

4.3.3 AMH Immunostaining

Detection of anti-mullerian hormone was carried out in order to provide comparative staining between the inhibin subunits and another member of the TGF- β superfamily highly expressed in the ovine ovary. AMH protein was seen in the majority of follicles in the ovary at all stages of development (Figure 4.7). Its

expression was confined to the granulosa cells only, with no staining for the protein observed in the theca layers above that of the control sections.

4.4 Discussion

This study has employed fluorescent immunohistochemistry to detect the expression of inhibin subunit proteins throughout the oestrous cycle, and dual fluorescent labelling to colocalise α and β B inhibin subunits within the ovine ovary in order to identify follicle populations capable of inhibin B production.

Expression of inhibin α -subunit was only noted in late preantral and antral follicles, supporting previous work inferring that antral follicles are the main source of inhibin in the sheep (Chapter 3; Mann *et al.*, 1992). Although preantral follicles are unlikely to produce inhibin due to low level expression of α subunit, the observation that small preantral follicles produce both β A and β B subunit protein is consistent with the possible production of activin.

However, as previously observed (McNatty *et al.*, 2000), β subunit mRNA was not detectable in primordial or primary follicles, suggesting that the source of the β subunit protein detected in these follicles lies elsewhere in the ovary. It is, however, possible that the *in situ* hybridisation method used in this work was not sensitive enough to detect low levels of mRNA possibly present in preantral follicles.

During analysis of the β B subunit staining, a high-level of protein was detected in the zona pellucida (ZP) of some preantral and early antral follicles. This staining is unlikely to be non-specific as similar patterns were not observed with the other antibodies utilised in this study. The high-level of staining in the ZP may be due to activin bound to activin receptors which are expressed by the oocyte in numerous species (Izadyar, *et al.*, 1998).

To the authors knowledge this is the first report of the presence of inhibin subunit proteins in the theca cell layers of the ovarian follicle. The staining for β -subunits seen in both the theca interna, externa and in some cases several cell layers into the stroma (Figures 4.1-4.4) indicates either the production of the proteins by thecal cells or their diffusion from the granulosa compartment. The fact that mRNA for both β -subunits was only ever witnessed in the granulosa cell layers supports the

hypothesis that activin may diffuse from the granulosa cells through the theca layers and into the surrounding tissues. The diffusion of activin in other tissues has been well researched (McDowell, *et al.*, 1997; Gurdon, *et al.*, 1999), showing that the dimer is able to travel up to seven cell lengths through embryonic *Xenopus* tissue. If this also occurs in the ovine ovary, activin may be able to act as an inter-follicular modulator. As there was no α -subunit staining observed in the theca externa, with a small amount seen in the theca interna of antral follicles, it may be suggested that entities containing the α -subunit do not diffuse to the same degree as those composed of β -subunits and that instead they are secreted into the blood via blood vessels in the theca externa. These observations may offer an explanation for the systemic action of inhibin compared to the mainly local action of activin.

The fact that both α and β B subunit proteins were colocalised to the granulosa cells of antral follicles (Figure 4.2) is consistent with the production of inhibin B by these follicles, which produce significant levels of inhibin A (Chapter 3). However, measurement of inhibin B in the plasma and follicular fluid of ewes is not possible using a specific assay (McNeilly, 2000) and therefore it is unlikely that if inhibin B is secreted by the ovary, that it is produced by the follicles.

No differences were seen in the expression of inhibin subunits at the three stages of the oestrous cycle studied in this work. Protein synthesis of the subunits seems to be dependant on size rather than the levels of hormones produced by the pituitary, this may be due to the influence of local ovarian factors during periods of decreased gonadotrophic support which are able to modulate the production of inhibin subunits (Tekmal, *et al.*, 1996; Bergh, *et al.*, 1997; Webb *et al.*, 1999).

The presence of immunostaining for β B subunit in the ovine *corpus luteum* (CL), and the distinct lack of staining for the other two inhibin subunits is consistent with the suggestion that the ovine CL does not produce mature inhibin (Mann, *et al.*, 1989). However, it is possible that the luteal tissue is able to produce activin instead of inhibin in contrast to the primate and human CL which have been shown to secrete considerable quantities of immunoreactive inhibin (Illingworth *et al.*, 1991). Activin produced by the luteal tissue in the ewe may play a role in the switch in cellular steroid production from oestradiol to progesterone induced by the surge levels of LH and possibly be involved in the extensive angiogenesis which is characteristic of luteal

development (Amselgruber, *et al.*, 1999).

The pattern of expression of AMH, seen in consecutive sections to those used for the inhibin subunit detection, supports the hypothesis that the β -subunit staining in the theca layers is diffusion and not an artefact. AMH, a member of the TGF- β superfamily along with inhibins and activins, showed no evidence of diffusion from the site of manufacture in the granulosa cells (Figure 4.7) (Bezard, *et al.*, 1988). However, AMH must diffuse in the fetal male to cause the degeneration of the Mullerian ducts (Price, *et al.*, 1977). It is possible that other members of the cohort of TGF- β related proteins may be able to diffuse through tissue in a similar manner; however, it is equally possible that the ovarian diffusion observed in this study is unique to activins. One possible explanation for the apparently selective diffusion of TGF- β proteins in the ovary is the make-up of the extracellular matrix (ECM) which varies considerably from tissue to tissue (Kantorova, 1994). It is possible that differences in the ECM allow the movement of certain molecules in certain tissues whilst preventing the transport of others leading to tissue specific diffusion, such as with AMH which diffuses in the fetal testis but not in the sheep ovary.

As to whether the effect of the diffusion of activin in the ovine ovary would be beneficial or detrimental to nearby follicles is uncertain. However, as large antral follicles seem to be a major source of activin, the protein may serve as an additional negative influence on the further recruitment of smaller follicles until the large follicles have either undergone atresia or ovulated, at which point the recruitment of another cohort of antral follicles is necessary. This hypothesis is supported by research in the rat showing that activin secreted by secondary follicles causes preantral follicles to remain quiescent *in vitro* (Mizunuma *et al.*, 1999).

In conclusion, this work has shown that there is differential expression of inhibin β subunit protein and mRNA in preantral follicles. The immunohistochemical staining has also detected diffuse β subunit staining but not mRNA expression in the theca cell layers of late preantral and antral follicles. It is hypothesized that activin, derived from these follicle populations acts as an intra-ovarian factor to modulate the growth and differentiation of subordinate follicles.

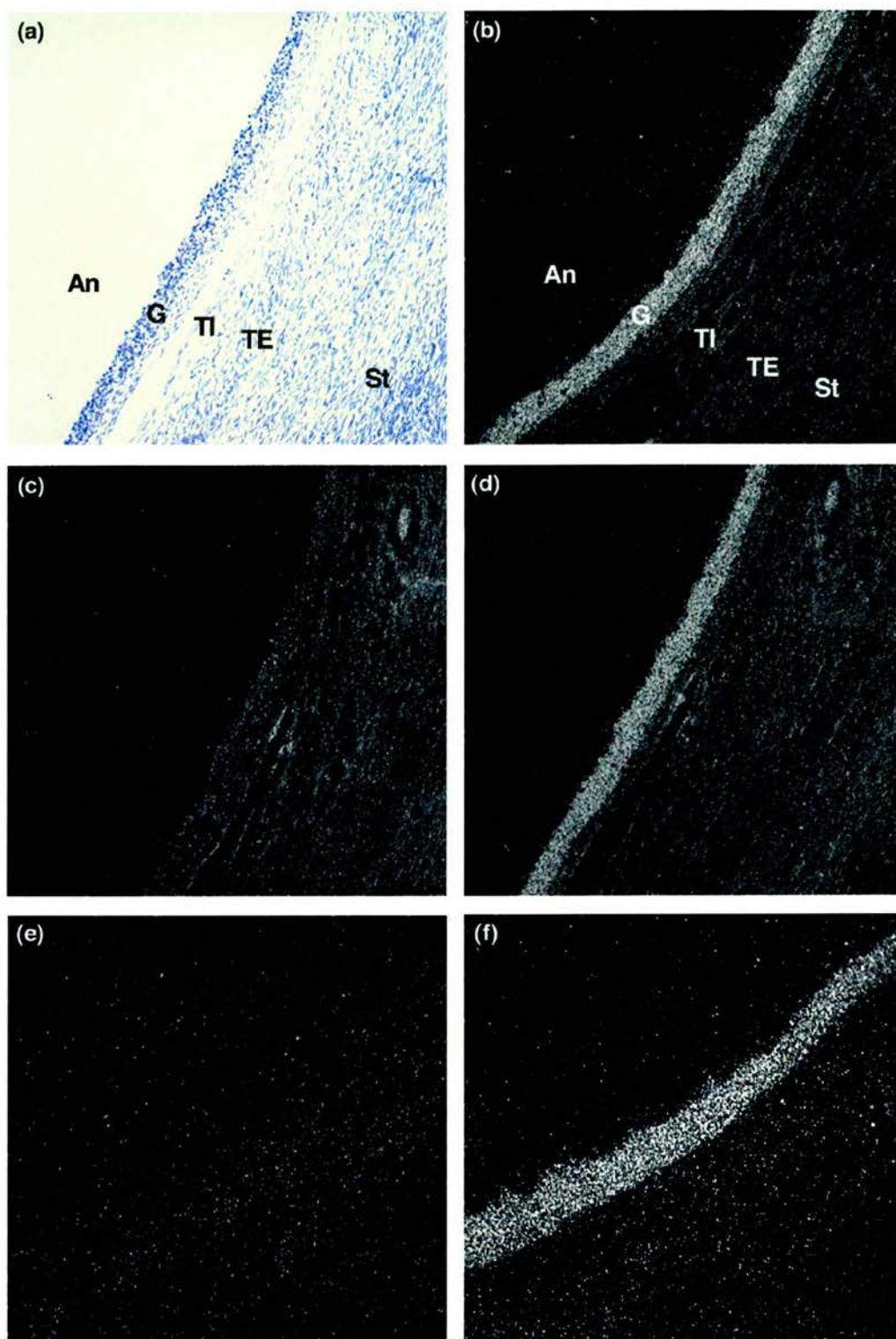


Figure 4.6 *In situ* hybridisation for inhibin β A (b), β B (d) and α -subunit (f) mRNAs in a large antral follicle (>3mm). Haematoxylin stained section (a) and sense controls (c, e). x100. An – Antrum; G – Granulosa; TI – Theca Interna; TE – Theca Externa; St – Stroma.

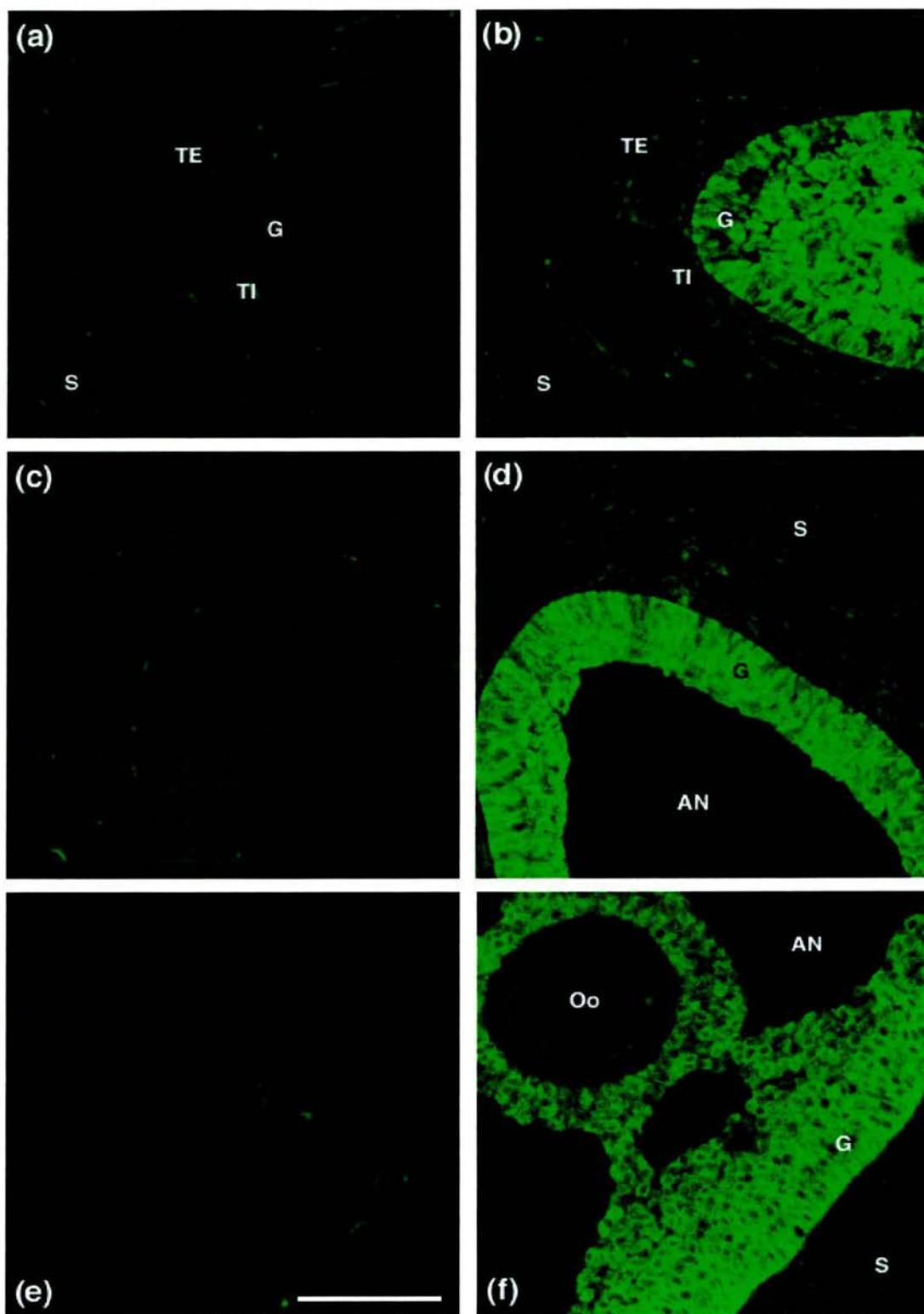


Figure 4.7 Immunostaining for AMH in large preantral (b), early antral (d) and medium antral (f) follicles. (a), (c) and (e) are control sections. S – Stroma; TE – Theca externa; TI – Theca interna; G – Granulosa; AN – Antrum; Oo – Oocyte. Scale Bar represents 100 μ m.

Chapter 5 – Forms of inhibins and activins synthesized by the ovine ovary

5.1 Introduction

Two types of inhibin (A and B) have been identified in a number of mammalian species. Although specific assays have been developed to measure both hormones in the human and the rat (Baly *et al.*, 1993; Fahy *et al.*, 1995; Groome, *et al.*, 1996; Robertson, *et al.*, 1997), so far only inhibin A is measurable in the sheep, using a modified version of the assay developed for human samples.

In human plasma, inhibin B peaks in the early follicular phase in contrast to inhibin A, which is low at this time. Concentrations then fall gradually during the follicular phase before peaking again shortly after ovulation and then declining for the rest of the luteal phase (Groome, *et al.*, 1996). The human *corpus luteum*, which secretes a considerable quantity of inhibin A (Illingworth, *et al.*, 1991), does not release inhibin B into the circulation (Illingworth *et al.*, 1996a). In addition, measurement of both forms of inhibin in human follicular fluid revealed concentrations of inhibin B 20-200 fold greater than those of inhibin A (Groome, *et al.*, 1996).

Research carried out using isolated rat granulosa cells has demonstrated the differential modulation of inhibin A and B production by steroids, gonadotrophins and local ovarian growth factors (Lanuza, *et al.*, 1999). Both FSH and IGF-1 alone stimulated the secretion of both inhibins with the release of inhibin A notably more responsive to FSH, and oestradiol only stimulating inhibin A production. In addition TGF- β brought about a considerable increase in the production of inhibin B that was three times that of inhibin A. This dual control of production supports the notion that the two forms of inhibin have a greater functional significance at differing stages of the oestrous cycle in rats and humans.

In addition to the measurement of inhibin B in the female, the hormone has also been detected in males of several species. In the human male, inhibin B is the predominant form in terms of the suppression of FSH, with inhibin A undetectable (Illingworth *et al.*, 1996b; Anderson *et al.*, 1997). An identical situation has been

shown in the rat (Woodruff *et al.*, 1996) where inhibin A is absent from the serum and inhibin B is the sole isoform of the hormone. In contrast, recent research has shown that the ram produces only inhibin A and not inhibin B (McNeilly, 2000). These experiments demonstrate a significant sexual difference in the forms of inhibin produced, with female mammals seemingly synthesizing both hormones and the males only producing one.

In addition to the two forms of 'mature' inhibin (32 kDa) there are a number of other isoforms of the precursor proteins which have been identified in several species. In the human a range of bioactive forms have been identified with molecular weights from 30 – 120 kDa (Robertson *et al.*, 1995), along with bio-inactive forms, 26 and 32 kDa in weight. In the cow, molecular weight inhibins of between 32 and 105 kDa have been detected (Sugino, *et al.*, 1992). Amino acid analysis of these proteins suggested that the 105 kDa protein is cleaved sequentially to finally give rise to the 32 kDa, or mature form of the hormone. The fact that these reports suggest that a number of the various forms of inhibins and activins identified (along with the mature forms of the proteins) have a significant biological activity, implicates them in the possible local control of folliculogenesis.

In chapter 4 it was shown that mRNA and immunoactive protein for inhibin α , β A and β B subunits were present in the granulosa cells of follicles in the sheep ovary. However, to date inhibin B has not been detected (using a specific enzyme-linked immunosorbent assay) in the peripheral blood or follicular fluid of the ewe (Knight, *et al.*, 1998; McNeilly, 2000).

A number of forms of inhibins and activins have been identified in the human (Robertson, *et al.*, 1995), pig (Guthrie *et al.*, 1997) and cow (Sugino, *et al.*, 1992). The aim of this work was to determine the types of inhibin and activin produced and secreted by the ovine ovary.

5.2 Materials and Methods

Ovine follicular fluids and follicle shells were analysed utilising Western blotting, in an attempt to identify whether the ewe produces inhibin B and if it does, whether it is secreted into the follicular fluid. We have detected different molecular

weight varieties of inhibin and activin subunits present in sheep follicles, employing the antibodies used in Chapter 4 of this thesis, in an attempt to describe the staining visualised by immunohistochemistry in the ovine ovary. Human follicular fluid (HFF) samples, which are known to contain significant levels of both inhibin A and inhibin B, were included on each Western gel in order to provide a positive control for the detection of inhibin B.

Materials

Ovarian ovine follicles used for this study were 1) large antral follicles (>4mm) collected from the ovaries of sheep slaughtered in an abattoir; 2) a range of follicles, luteinised follicles and *corpora lutea* (mid-cycle) collected from the ovaries of Scottish Blackface ewes in the breeding season (see Chapter 3); and 3) follicular fluid collected from the first flush of large antral human follicles collected from *in vitro* fertilization cycles as a positive control for inhibin-B (IVF Unit, Simpson's Maternity Hospital, Edinburgh, UK).

Methods

5.2.1 Ovariectomy and Follicle dissection

Anaesthesia was induced by minimal quantities (200-300 mg) of a mixture of two parts thiopentone (Intraval; RMB Animal Health Ltd, Dagenham, Essex, UK) and one part pentobarbitone sodium (Sagatal; May and Baker Ltd, Dagenham, Essex, UK) and maintained using halothane (1.5 – 2.5%). Mid-ventral laparotomy was then performed and the reproductive tract exteriorised.

The ovaries were then removed and both ovaries from two animals in each group were fixed for histological analysis. Ovaries from the remaining 18 sheep were microdissected and all visible antral follicles collected. Follicle diameter was recorded (in mm) prior to each follicle being placed in an individual well of a 24 well culture plate in 1 ml of Medium 199 (Sigma, Poole, Dorset, UK) and hemisected using microscissors. Finally, one half of the follicle shell was fixed in paraformaldehyde and the other half snap frozen in liquid nitrogen.

5.2.2 Primary antibodies

Immunochemical detection from the western blots was carried out using a rabbit polyclonal antibody raised against porcine inhibin α -subunit (Professor A. S. McNeilly, Human Reproductive Sciences Unit, Edinburgh, UK) along with mouse monoclonal antibodies raised against β A subunit (E4 clone) and β B subunit (12/13 clone) (Professor N. Groome, Oxford Brookes University, UK). Additionally, a mouse monoclonal antibody raised against the Pro- β A subunit of inhibin was also used for protein detection (A. Tsigos, Oxford Brookes University, UK).

5.2.3 Western Blot (Please see appendix for detailed protocol)

Tissues were homogenised in protein extraction buffer (20mM Tris-HCl, 150mM NaCl and protease inhibitor cocktail – Boehringer Mannheim). The concentration of protein in undiluted and 1:10 diluted samples was measured at least three times using a Genequant Pro (Amersham Pharmacia Biotech) and the homogenate concentration adjusted with extraction buffer to provide a protein concentration of 1mg/ml. 20 μ l of each sample was mixed with 5 μ l of sample application buffer with β -mercaptoethanol present (reducing) or absent (non-reducing). Samples were loaded onto 15 well 4 – 20% tris-glycine gels (Novex) and separated at 120V, 26mA for 2 hours. Electrophoresed proteins were transferred to polyvinylidene fluoride (PVDF) membranes in transfer buffer (192mM Glycine, 25mM Tris Base, 20% Methanol and 0.1% SDS) at 30V, 400mA for 2 hours. Gels were stained with Coomassie blue solution for 5 minutes. Membranes were blocked with 5% porcine serum in TBS-T (50 mM Tris-HCl, 150mM NaCl, 0.05% Tween, pH 8.0) for one hour and incubated with primary antibody (1:1000 dilution in blocking solution) overnight at 4°C. After repeated washing with TBS-T the membranes were incubated with alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgGs for one hour at room temperature. After further washes with TBS-T, the membrane was coated with enhanced chemifluorescence (ECF) alkaline phosphatase substrate (Amersham Pharmacia Biotech) and staining detected using a Storm Phosphoimager at 800V.

5.3 Results

The Western blots presented in this Chapter are in each case representative of a number of gels on which identical samples have been run out under the same conditions with the same bands consistently detectable with specific inhibin antibodies.

5.3.1 Inhibin subunit isoforms in the cells of additional ovarian structures

In order to investigate whether inhibin B is present in the ovine ovary, Western blot analysis was carried out using a number of different tissues collected from ovaries supplied by a local abattoir, including a range of antral follicles, *corpora lutea* (CL) and luteinised follicles (Figure 5.1).

Utilising an antibody raised against mature inhibin α subunit, a number of bands from 95 to >200 kDa were detected under non-reducing conditions in the follicle shells (granulosa and theca cells) of large (>4mm) follicles, together with another series of bands between 40 and 47 kDa. Reduction of these samples produced bands of 25, 31 (only in >4mm), 35, 42 and 47 kDa in follicle shells from all healthy follicles, with the intensity of the bands increasing with follicular size.

Detection with a β A-subunit antibody revealed a pattern of bands in large follicles similar to that detected with the α subunit antibody. In non-reducing conditions bands of >90 and 46 kDa were observed with reduction of these proteins leading to bands of 38, 42 and 49kDa. Incubation of the same blot with an antibody raised against the β B subunit again produced a range of protein bands comparable with those containing the mature α subunit sequence. Prior to reduction, proteins with molecular weights of >95 and 46kDa were detected in large antral follicles and addition of β -mercaptoethanol to the protein extracts produced bands of 36, 38, 42, and 49kDa.

Human follicular fluid, included as a positive control for both inhibin A and B, contained α subunit related proteins of 70 - >110 kDa which were cleaved to produce 31 and 49 kDa bands in addition to the original 70 - >110 kDa entities. The same samples contained β A-associated proteins of 29, 47 and 90 kDa in non-reducing conditions which were unaffected by reduction. Also present in HFF were two non-

reduced β B subunit-containing bands of 29 and 90 kDa, which were also present in the reduced sample.

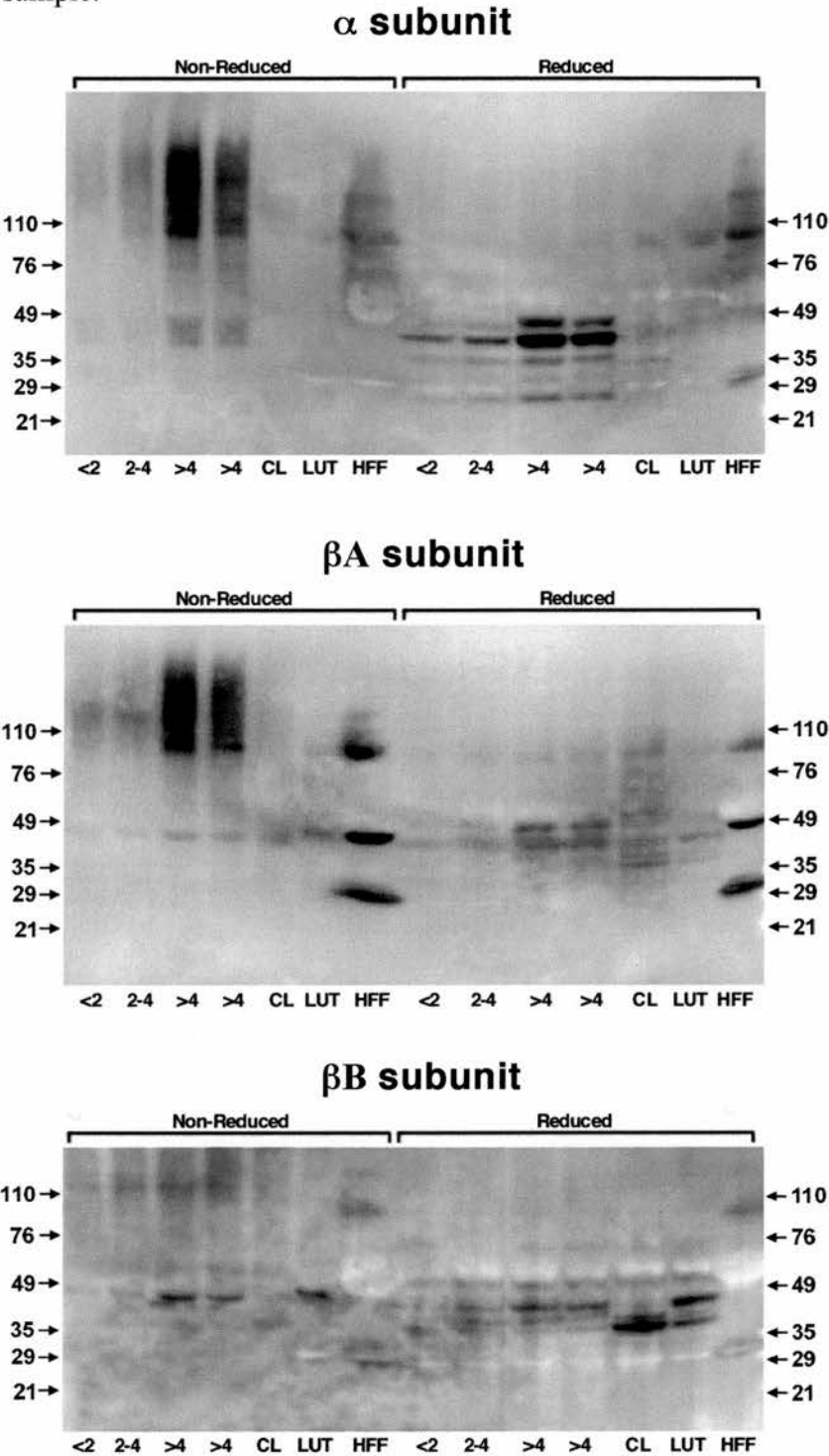


Figure 5.1 Immunodetection of inhibin subunit-associated molecules in a range of ovarian structures. CL – *corpus luteum*; LUT – Luteinised follicle; HFF – human follicular fluid. (<2, 2-4, >4 – follicle diameter in mm). Protein marker weights; 21 – 110 kDa.

5.3.2 Inhibin subunit isoforms in the shells of large follicles

In order to examine further those β B associated proteins observed in large antral follicles, and in an attempt to gain further evidence for the presence of inhibin-B in the cells of these follicles, a Western blot was performed on follicle shells collected from a number of large ovine antral follicles (>4mm) dissected from abattoir ovaries (Figure 5.2).

When the blot was incubated with an antibody raised against the α -subunit, a clear band of 46 kDa together with a series of bands between 76 and >110 kDa were seen in non-reduced samples. When these samples were reduced, a number of new bands were observed due to the cleavage of any disulphide bonds linking protein molecules. Proteins with molecular weights of 26, 38 (only present in follicles 1 and 5), 45 and 49 kDa were observed in these reduced samples.

Incubating the same blot with an antibody raised against inhibin β B subunit revealed faint bands of 44 and 52 kDa in weight prior to reduction and 44 and 50 kDa in reduced samples.

Bands present in the HFF were not as clear as those observed in previous blots, however, α -subunit associated proteins of >90 kDa were observed in non-reducing conditions, with proteins of 28, 47 and >90 being detected in reduced samples. 90 and 115 kDa β B-associated proteins were observed in non-reducing conditions in HFF, with proteins of 30, 34, 45 and 90 kDa being produced upon addition of a reducing agent.

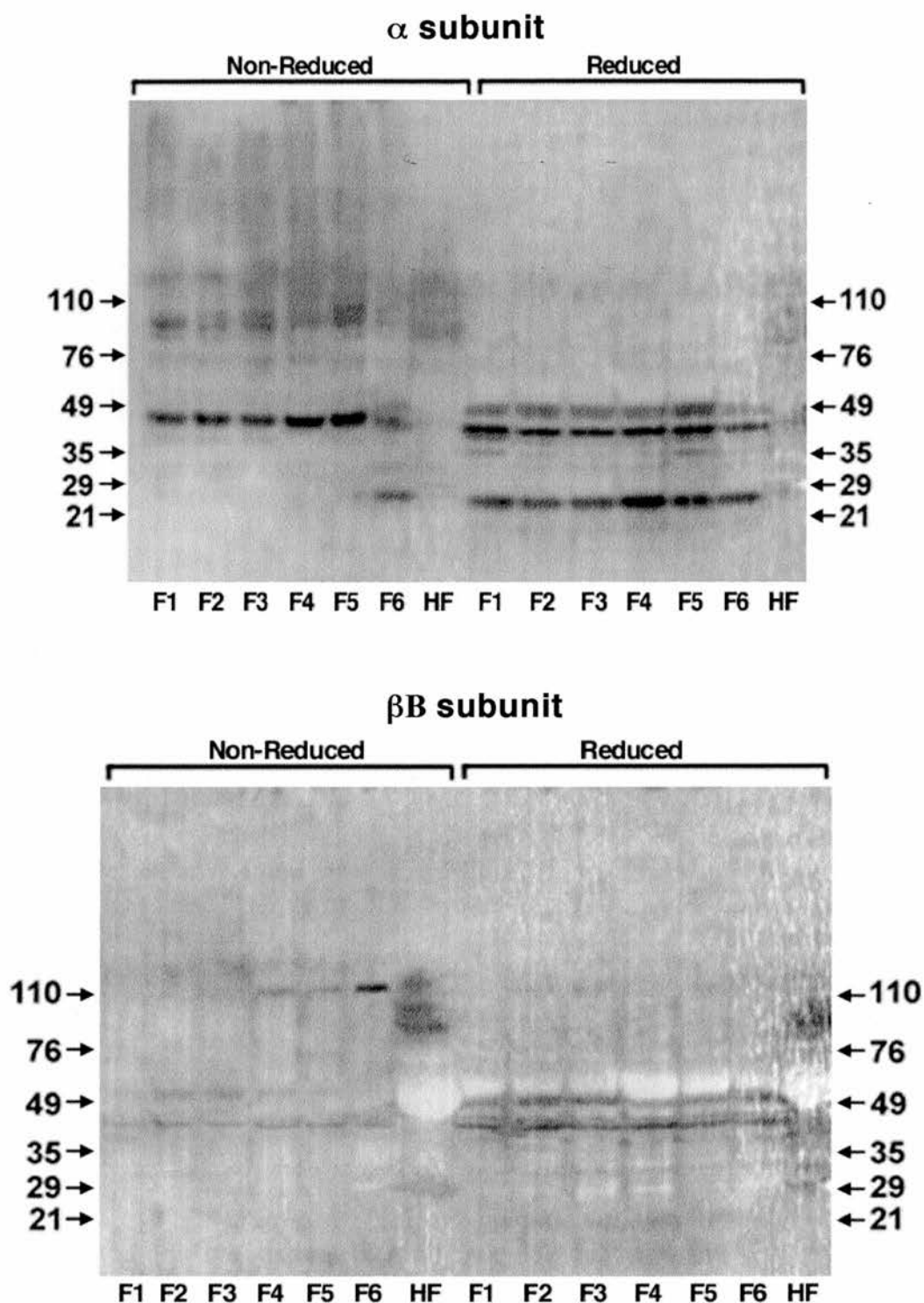


Figure 5.2 Detection of inhibin α and β B subunit-associated molecules in the follicular cells of large (>4mm) ovine antral follicles. F1-6 – ovine follicle shells; HF – human follicular fluid. Protein marker weights; 21 – 110 kDa.

5.3.3 Inhibin subunit isoforms in ovine follicular fluid

Western blotting of follicle shell samples gives an indication of those subunit forms produced by the cells of the follicle. In order to investigate the inhibin-related proteins secreted by the cells into the follicular fluid, Western blots were carried out using follicular fluid samples collected from large antral follicles (Figure 5.2) supplied by a local abattoir and previously described in this Chapter (Section 5.3.2).

Detection of α -subunit associated molecules in these follicular fluids (Figure 5.4) revealed a collection of bands greater than 100 kDa in weight, along with a strongly stained 45 kDa band. The addition of a reducing agent produced three bands of 22, 45 and 49 kDa. Incubation with the β A antibody detected non-reduced proteins of ~16, 27, 32, 42, 49-60 and >110 kDa in ovine follicular fluid. Reduction of these samples seemed to increase the intensity of the 49 kDa band, however, the lighter 16 – 42 kDa bands were not detected in the reduced samples.

There were no β B-associated proteins detected in either non-reducing or reducing conditions in ovine follicular fluid. In contrast, HFF contained a range of bands >100 kDa which were immunoreactive with all three inhibin subunit antibodies. When these samples were reduced, a 90 kDa protein was detected with all three antibodies. This band was considerably lighter when detected with the α -subunit antibody, and a range of bands from 50 - >110 kDa were observed. In addition to the 90 kDa band, both β -subunit antibodies detected proteins of 31 and 33 kDa, with the β A antibody also revealing a 50 kDa band.

Along with the detection of molecules containing the protein sequence found in the three mature inhibin subunits, another antibody raised against the Pro- β A subunit was employed in an attempt to define which forms of the β A subunit have been detected. This antibody does not bind to mature β A subunit or mature inhibin A.

Incubation with this antibody detected bands of 33, 43, 46, 55, 90 and >110 kDa (Figure 5.5). Addition of β -mercaptoethanol to the samples gave rise to bands of 34 and 48 kDa. In contrast, there were no clear bands evident in HFF in either non-reducing or reducing conditions.

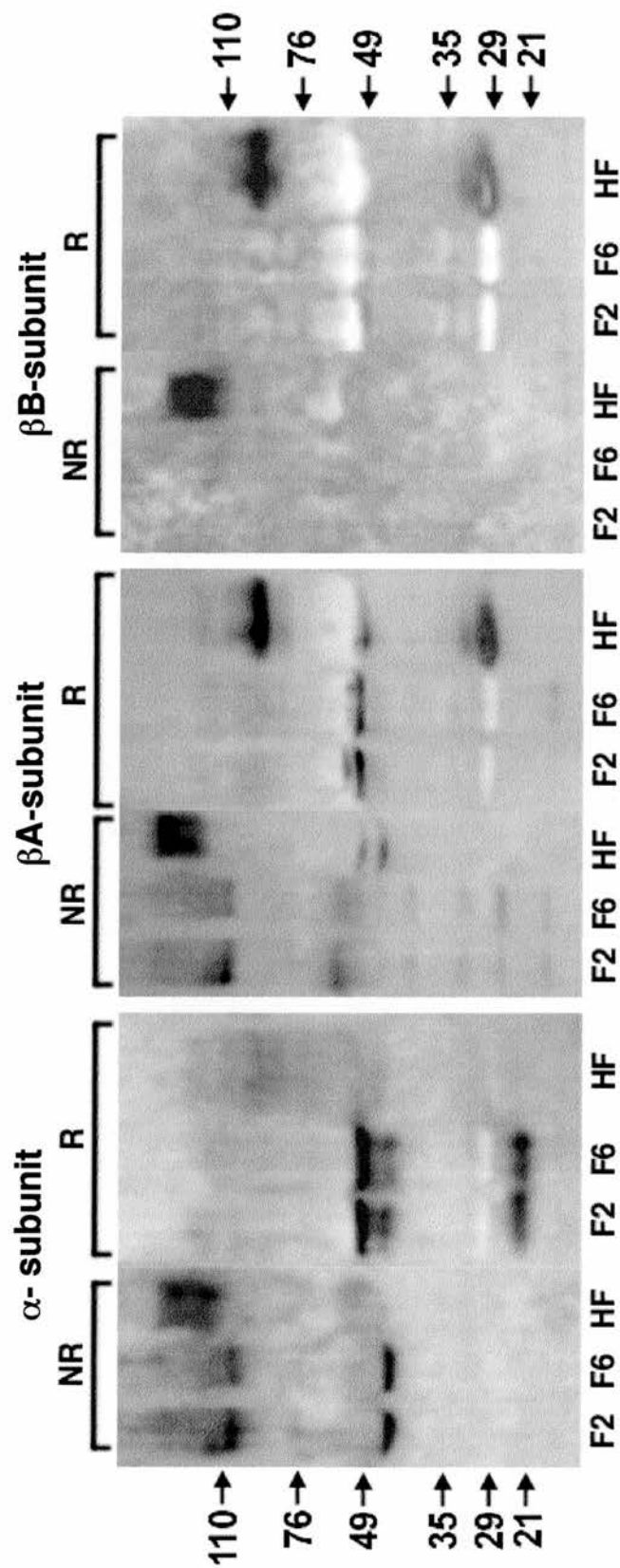


Figure 5.3 Immunodetection of α , β A and β B associated proteins in the follicular fluids of large ovine (F2, F6) and large human (HF) follicles. NR – Non-reduced; R – Reduced; HF – Human follicular fluid. Protein marker weights; 21 – 110 kDa.

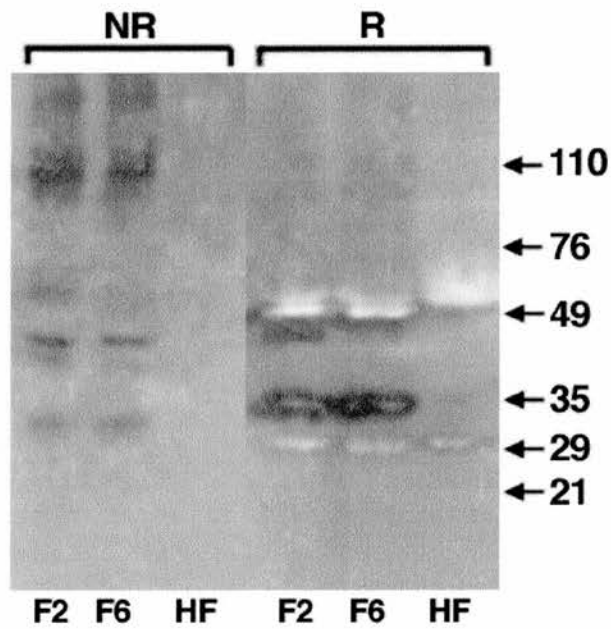


Figure 5.4 Detection of inhibin Pro- β A associated molecules in follicular fluid from large ovine (F2, F6) and large human (HF) antral follicles. NR – Non-reduced; R – Reduced; HF – Human follicular fluid. Protein marker weights; 21 – 110 kDa.

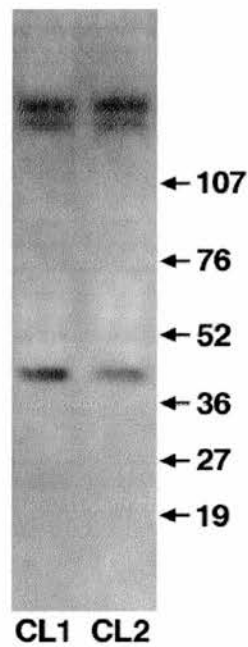


Figure 5.5 Immunodetection of inhibin β B-subunit associated molecules in luteal tissues. CL – *corpus luteum*. Protein marker weights; 19 – 107 kDa.

A faint band of approximately 40 kDa was visible in the mid-cycle CL (Figure 5.1), and in order to clarify this protein another blot was performed (Figure 5.5), revealing a clear band of 42 kDa with additional bands >107 kDa.

5.4 Discussion

Healthy ovine antral follicles have been shown to produce and secrete immunoreactive inhibin A (Chapter 3) and as expected express both α and β A subunits in their granulosa cells. The granulosa cells of these follicles also express a large amount of β B subunit protein (Chapter 4) which is required for the production of both activin (B and AB forms) and inhibin B. In addition, the granulosa cells of most antral follicles also express mRNA for α , β A and β B subunit with β B staining almost always present in large healthy follicles (Engelhardt, *et al.*, 1993).

Therefore, the main aim of this study was to attempt to discover whether ovine follicles, or other ovarian structures produce inhibin B.

Proteins of 46 and >90 kDa containing the mature α and β B subunit sequences were identified in the granulosa/theca cells of antral follicles (Figure 5.1). These bands are compatible with the production of inhibin B forms by the ovine ovary. However, the weights of these proteins are consistent with inhibin B precursor dimers (>45 kDa) and not the mature form of the heterodimer (~32 kDa). In addition to the mature 32 kDa form of inhibin A, non-cleavable forms of inhibin A precursor proteins are capable of suppressing FSH secretion in a rat pituitary cell bioassay (Mason, *et al.*, 1996). Therefore, if high molecular weight forms of inhibin B exhibit this bioactivity, and are present in the ovary, it is possible that they may exert some form of local autocrine or paracrine effect. Furthermore, recent research has shown that levels of certain high molecular weight forms of inhibin A may be more responsive to FSH than others (Boudjemaa, *et al.*, 2000), suggesting possible differential control of molecular weight forms of inhibin within the ovary.

If inhibin B produced by the follicle cells has a systemic role in the ewe, the presence of the hormone in the follicular fluid of ovarian follicles would be expected, as with inhibin A (Chapter 3). This hypothesis is supported by the fact that high levels of inhibin B are found in human follicular fluid with levels 20- to 200- fold greater than inhibin A (Groome, *et al.*, 1996). Examination of Western blots carried

out using follicular fluids from large antral follicles (Figure 5.3) revealed no β B subunit-associated proteins in the follicular fluid of ovine follicles. This was in striking contrast to the numerous α and β A containing proteins detected in the same samples. In addition, β B subunit bands (along with α and β A) were observed in human follicular fluid, included as a positive control for inhibin B. It is unlikely that the lack of β B detection in ovine follicular fluid is due to a technical factor as the antibody used specifically detects the subunit in the human and also specifically detects an ovine protein using histochemical methods (Chapter 4). Given the very high level of homology between the β B subunits of the human, sheep and other mammalian species it is highly unlikely that the antibody used is non-specific and these data therefore provide evidence that inhibin B forms are not secreted into the follicular fluid of large ovine antral follicles by the follicular cells. Furthermore, the lack of any proteins detectable with an anti- β B subunit antibody in ovine follicular fluid, suggests that activin forms containing β B subunit are not secreted by follicles of this size. This hypothesis contradicts the detection of high levels of activin AB in ovine follicular fluid by a specific two-site ELISA (Evans, *et al.*, 1997).

The follicular fluids of smaller antral follicles were not examined in this study, as blotting and immunodetection of their follicle shells revealed lower levels of the β B associated proteins compared to large antral follicles. As the cells of the follicle are assumed to be the site of manufacture of inhibin subunits, the follicular fluids of large antral follicles were selected as the most likely source of any secreted inhibin B.

When the pattern of non-reduced and reduced bands observed in the follicular fluid of the ewe is compared to inhibin forms identified in other species, there are a number of parallels between the proteins detected. In both bovine and porcine follicular fluid an α subunit containing band of ~46 kDa is present along with a range of bands >60 kDa (Guthrie, *et al.*, 1997). After treatment with a reducing agent, proteins of 23, 47 and 59 kDa were detected in both species. These bands may well be consistent with the non-reduced 45 and >90 kDa, and reduced 22, 45 and 49 kDa bands observed in this study. The fact that additional non-reduced bands of 27 and 32 kDa together with a reduced band of 29 kDa were present in the cow and pig, but not the ewe, may reflect their absence in ovine follicular fluid or possibly a reduced

level of protein purification in this study. However, these observations do suggest the presence of a number of inhibin subunit/dimer forms with comparable molecular weights in a number of species.

A faint β B protein of ~40 kDa was evident in the CL (Figure 5.1) and a further blot with non-reduced CL samples (Figure 5.5) revealed a more visible β B-containing protein of 42 kDa and additional high molecular weight bands (>107 kDa). These proteins are likely to represent the staining observed in the CL in Chapter 4 of this thesis.

These findings are surprising because the mature ovine *corpus luteum* does not secrete immunoactive inhibin (Mann, *et al*, 1989; Bramley, *et al*, 1992) and does not express mRNA for α , β A or β B subunit (Engelhardt, *et al.*, 1993; Tisdall, *et al.*, 1994). However, mRNAs for inhibin α and β A subunit have been observed in luteal tissue shortly after ovulation (Engelhardt, *et al.*, 1993) with the level of expression becoming undetectable as the CL matures. The CL samples were included in the present study as negative control tissue as the expression of any inhibin subunits had not been observed previously. The fact that mRNA for β B subunit is not expressed in the sheep CL, and β B-associated proteins have been detected in this tissue, is consistent with the hypothesis that the origin of these proteins lies elsewhere in the ovary, possibly neighbouring antral follicles. It may, however, be possible that the *in situ* hybridisation protocol used for this research was less sensitive than the immunocytochemical method and thus low levels of mRNA may have remained undetected.

Furthermore, in reduced follicular shell samples, a 36 kDa β B-associated protein remained unchanged throughout follicular development but seemed to be increased considerably in the luteinised follicle and then further increased in the CL. In contrast a reduced 42 kDa protein was increased during follicular development and hardly detectable in the CL. These observations may signify a switch in the processing of the β B subunit during the process of CL development

It is possible that bands detected with molecular weights of >65 kDa in both the follicle shells and follicular fluids of both species studied are a mixture of both precursor dimers constituting high molecular weight inhibin and activin forms, along

with various forms of inhibin and activin bound to follistatin (FS). These complexes have been identified in the human and have molecular weights between 66 and >220 kDa (FS-inhibin) and in the case of a FS-activin complex, a weight of around 97 kDa (Wang, *et al.*, 1999).

Intensities of bands seen in ovine follicles were greater in large antral follicles than those in small follicles, possibly reflecting the predicted increase in the production of inhibin subunits with follicle size (Chapter 3 and 4).

Finally, the presence of β B staining observed in the *corpus luteum* (Chapter 4) along with the detection of β B subunit (and not α or β A) containing proteins in CL tissue via Western blotting, indicates the possible production of activin B by ovine luteal tissue. However, as mRNA for β B subunit has not been observed in the ovine CL it is likely that these proteins are the product of other ovarian structures, possibly suggesting paracrine interactions between follicles and the CL.

In summary, these findings, which demonstrate the existence of protein bands binding both α and β B subunit antibodies, are compatible with the presence of inhibin B dimers in follicular cells. However there is a possibility that rather than a band being one distinct protein, it may be several of comparable weights, in this case possibly an activin precursor and a form of α subunit, and not an inhibin dimer.

Additionally, this research provides evidence that β B subunit proteins are not secreted into the follicular fluid and it is possible that the β B proteins detected in the follicle shells represent a percentage of the diffused β B staining observed in the theca cell layers in Chapter 4 of this thesis.

To conclude, this work suggests that the subunits required for the production of inhibin B are present in the follicular cells of ovine antral follicles, and more significantly that proteins present in the granulosa/theca cells contain forms of both α and β B subunits, suggesting the presence of inhibin B dimers. However, the absence of β B subunit proteins in the follicular fluid suggest that if inhibin B protein is produced by granulosa cells it is not secreted into follicular fluid.

Chapter 6 - Effect of the Fec^B gene on the expression of inhibin subunits during follicular development

6.1 Introduction

In order to investigate further the role of inhibins and activins in folliculogenesis in the ewe, it is useful to examine breeds of sheep which exhibit an increased ovulation rate, as these animals are likely to show variations in the levels of certain factors involved in the control of follicles development (Baird and Campbell 1998).

An example of such a population are sheep carrying the fecundity gene (Fec^B) which have an increased ovulation rate compared to non-carrier animals. Over the years numerous studies have recorded differences in sheep carrying this gene with respect to factors known to influence the process of folliculogenesis. These include a reported increase in the level of FSH and LH in the peripheral circulation of carrier animals (McNatty and Henderson, 1987; Later contradicted by Souza, *et al.*, 1997b), decreased incidence of follicular atresia (Mandiki, *et al.*, 2000) and decreased IGFBPs in antral follicles of Fec^B animals leading to an increase in bioavailable IGF (Monniaux, *et al.*, 2000).

Although the gene responsible for the phenotype witnessed in these animals has been identified, the factor for which the gene codes has remained elusive until recent work identified a mutation in a specific receptor in these animals (Souza, *et al.*, 2001; Wilson, *et al.*, 2001; Mulsant, *et al.*, 2001) The nature of this mutation is discussed in detail in Chapter One of this thesis.

The effect of the Fec^B gene is widespread in the context of follicular development, with elements affected at many different stages of the process. However, as yet, the subunit expression of inhibin and activin, factors known to influence FSH release from the pituitary and thus follicle growth, has not been researched in these animals.

Measurement of inhibin A in the plasma of carrier and non-carrier ewes shows no difference between the secretion rate of the hormone into the circulation, as well as comparable levels of progesterone, androstenedione and oestradiol between genotypes (Souza, *et al.*, 1997b). Furthermore, the highest amounts of inhibin

produced by granulosa cells isolated from both genotypes are comparable, and are derived from large, healthy follicles in both groups of animals (Henderson *et al.*, 1991). Although the Fec^B mutation does not seem to reduce the level of inhibin or oestradiol feedback to the pituitary, which would increase FSH release, differences in inhibin and activin expression may play a role within the ovaries of these animals. Additional studies have suggested that although the level of inhibin in the circulation is not significantly different in Fec^B ewes, the stability of the mRNA coding for the βA subunit is increased in these animals, possibly increasing local inhibin A/activin production in the ovary (Fleming *et al.*, 1992).

The aim of this work was to examine the expression of the three inhibin subunits α , βA and βB in the ovaries of Booroola animals carrying the Fec^B gene and to compare the patterns of expression with non-carrier ewes in an attempt to identify any role these proteins might have in the premature development of ovulatory follicles in these sheep.

6.2 Materials and Methods

6.2.1 Ovarian Tissue

Ovaries were removed during the breeding season from 6 homozygous wildtype (++) and 5 homozygous FF (Fec^B gene) 22 month old, Scottish Blackface Merino cross ewes, in which the genotype was determined by pedigree (Haley, 1990). For each animal one ovary was microdissected as part of an unrelated study and the other was fixed overnight in 4% paraformaldehyde before being embedded in paraffin wax and sectioned as described in Section 2.7 of this thesis. Sections of the tissue were then taken at a thickness of 5 μ m and transferred to individual slides prior to immunohistochemistry. It should be noted that the fact that only one ovary per animal was fixed for this work may explain the low number of antral follicles observed in total in these animals.

6.2.2 Primary Antibodies

The antibodies targeted against the α , βA and βB subunits of inhibin used in this study are as previously described in Chapter 4 of this thesis.

6.2.3 Immunohistochemistry

Tissue sections from animals of both genotypes were incubated under identical conditions, in the same run of slides, using the same preparations of antibodies for both ++ and FF tissue. The immunohistochemistry protocol is identical to that described in Chapter 4 of this thesis.

6.2.4 Statistical Analysis

Measurement of staining intensity was recorded using Scion Image analysis software (Scion Corp, USA) using the method of observation described earlier in this thesis (Chapter 2). This method of measuring staining intensity was validated as described in section 2.8.3 of this thesis. Differences in levels of staining between genotypes were identified using a standard two-tail *t*-test. The mean number of follicles examined was 8.6 ± 0.7 follicles/class for the wildtype animals and 8.2 ± 0.9 follicles/class for the Booroola ewes. Follicles were grouped using the classification system described in section 4.2.7 of this thesis.

6.3 Results

As observed previously in this thesis (Chapter 4), the pattern of inhibin subunit expression was closely associated with follicle development. Proteins containing α -subunit were found in the granulosa cells of late preantral and antral follicles with both β subunits present in the granulosa compartment of follicles at all stages of development. In addition, β -subunit staining was evident in the theca cell layer of late preantral and early antral follicles in both groups of animals.

In general the levels of inhibin subunit staining measured were comparable between the two genotypes studied, however, a number of significant difference were noted between the two groups of animals.

The levels of α subunit protein in the granulosa cells of medium sized antral follicles were significantly lower ($P < 0.01$) in FF animals than those seen in the non-carrier ewes (Figure 6.1). It should be noted that this was the only difference in the level of α subunit seen between the two genotypes in follicles of a comparable size (Figure 6.3 – a, b).

The pattern of staining recorded for β A subunit protein in granulosa cells was very similar to that observed for the α subunit, with the levels of staining only significantly lower ($P < 0.05$) in the FF ovaries in medium sized antral follicles than those from ++ animals (Figure 6.3 – c, d).

However, this pattern was not evident when detecting β B subunit, with the only difference between the two groups of animals being an increased level ($P = < 0.05$) of the protein in the granulosa cells of late preantral follicles from FF ewes, compared to the non-carrier group.

Examination of the subunit staining seen in the theca cell layers also revealed a number of differences between the two groups (Figure 6.2). The low level of α subunit staining seen in the granulosa cells of medium sized antral follicles was mirrored in the theca layer, where a lower level ($P < 0.05$) was also observed in the FF ovaries.

In contrast to the difference seen in the β A subunit staining in the granulosa cells, there were no significant variations in the expression of this protein in the theca cell layers.

Although there were no significant differences recorded in antral follicles between the two genotypes, with respect to β B subunit protein, levels were significantly higher in the theca cells of both medium and large antral follicles in the FF animals compared to those in the ++ group (Figure 6.3 – e, f).

6.4 Discussion

The phenotype of ewes homozygous for the Fec^B gene is an increased ovulation rate due to precocious development of ovulatory follicles. As previously discussed, this has been attributed to variations in a number of factors relating to folliculogenesis. Although the mutation of the BMP receptor type 1B has recently been reported as the origin of the genotype (Souza, *et al.*, 2001; Wilson, *et al.*, 2001), the molecular mechanisms involved in the early development of ovulatory follicles in these animals is not fully understood.

Recent research strongly suggests that the mutated gene in FF animals influences ovarian rather than pituitary function. When animals were rendered hypogonadotrophic by the administration of GnRH antagonist, subsequent infusion

of physiological amounts of FSH and LH caused no reduction in the ovulation rate, indicating that increased FSH levels are not required for the precocious maturation seen in these animals and that the *FecB* gene acts at the level of the ovary (Campbell, *et al.*, 1996b).

The fact that medium sized antral follicles express lower levels of both the α and βA subunits in FF animals compared to ++ ewes may lead to a decrease in the production of inhibin A, or indeed activin A, by these follicles. This hypothesis is supported by the fact that α -subunit was also lower in the theca interna cells of medium antral follicles reflecting a decrease in the diffusion of the protein from the granulosa cells and thus production in general. This suspected decrease in inhibin A production from medium antral follicles in carrier animals may not be detectable in the circulation when compared to ++ animals, as although this class of follicle may produce less inhibin A there are a greater number of them in the ovaries of carrier ewes, so the total ovarian secretion of the hormone is comparable.

In contrast to these findings, previous research examining the expression of inhibin subunit mRNAs in the ovaries of *FecB* carrier and non-carrier ewes, has shown increased levels of both aromatase and inhibin βA subunit mRNA in medium antral follicles (Campbell *et al.*, 1998b). These observations concur with the precocious maturation of ovulatory follicles in this species, with medium antral follicles having increased levels of the proteins required for oestradiol and inhibin A production, characteristic of larger antral follicles in non-carrier ewes. Therefore, the decreased level of α and βA subunit protein seen in medium antral follicles in carriers of the *FecB* gene may be due to increased release of the subunits into the follicular fluid or the blood.

The granulosa cells of late preantral follicles had greater levels of βB subunit in FF animals than wildtype animals, suggesting a possible increase in the production of activin from these follicles. It is unlikely that this βB staining represents inhibin B as α subunit is not detectable in these follicles (Chapter 5).

Levels of βB subunit in the theca cells were maximal in medium and large antral follicles in animals homozygous for the *Fec^B* gene. Increased diffusion of βB subunit

containing proteins may reflect an increase in the production of activin by these follicles.

Although elevated FSH levels have been reported in animals carrying the *FecB* gene (McNatty and Henderson, 1987), further studies have observed no difference in the secretion of gonadotrophins (Souza, *et al.*, 1997b). This may suggest that an increase in activin may amplify the effect of FSH within the *Fec^B* ovary, rather than concentrations of the gonadotrophin being affected.

To conclude, we have noted an apparent decline in the expression of both α and β A subunit protein in the granulosa cells of medium sized antral follicles in animals homozygous for the *Fec^B* gene compared to non-carrier ewes. This may reflect a decrease in the output of inhibin A from each individual follicle, however, the levels of inhibin secreted into the periphery do not vary between FF and ++ animals (Souza, *et al.*, 1997b) suggesting that this possible decline in production may be of local significance within the ovary. It is unlikely, however, that a fall in inhibin A release by these follicles could be responsible for the phenotype of the Booroola animals, as there is considerable evidence supporting the hypothesis that the *Fec^B* mutation results in alterations in the BMP signalling pathway (Mulsant, *et al.*, 2001; Souza, *et al.*, 2001; Wilson, *et al.*, 2001), which in turn result in a premature induction of follicle cell differentiation. Therefore, it is likely that the decline in α and β A subunit expression in these follicles is a consequence of the mutation and not a cause.

Additionally, the higher levels of β B subunit expression noted in the medium sized and large antral follicles of Booroola animals are also likely to be a result of the presence of the *Fec^B* gene. It should be noted that if the β B subunit staining observed represents activin, there may be increased responsiveness of these follicles to FSH as activin has been shown to up-regulate FSH receptor expression and enhance cellular responsiveness to FSH (Xiao, *et al.*, 1992). Therefore, although the apparent increase in β B subunit diffusion from certain follicles in Booroola animals may not be a direct cause of the phenotype, it is possible that it may well amplify the effect of the mutation.

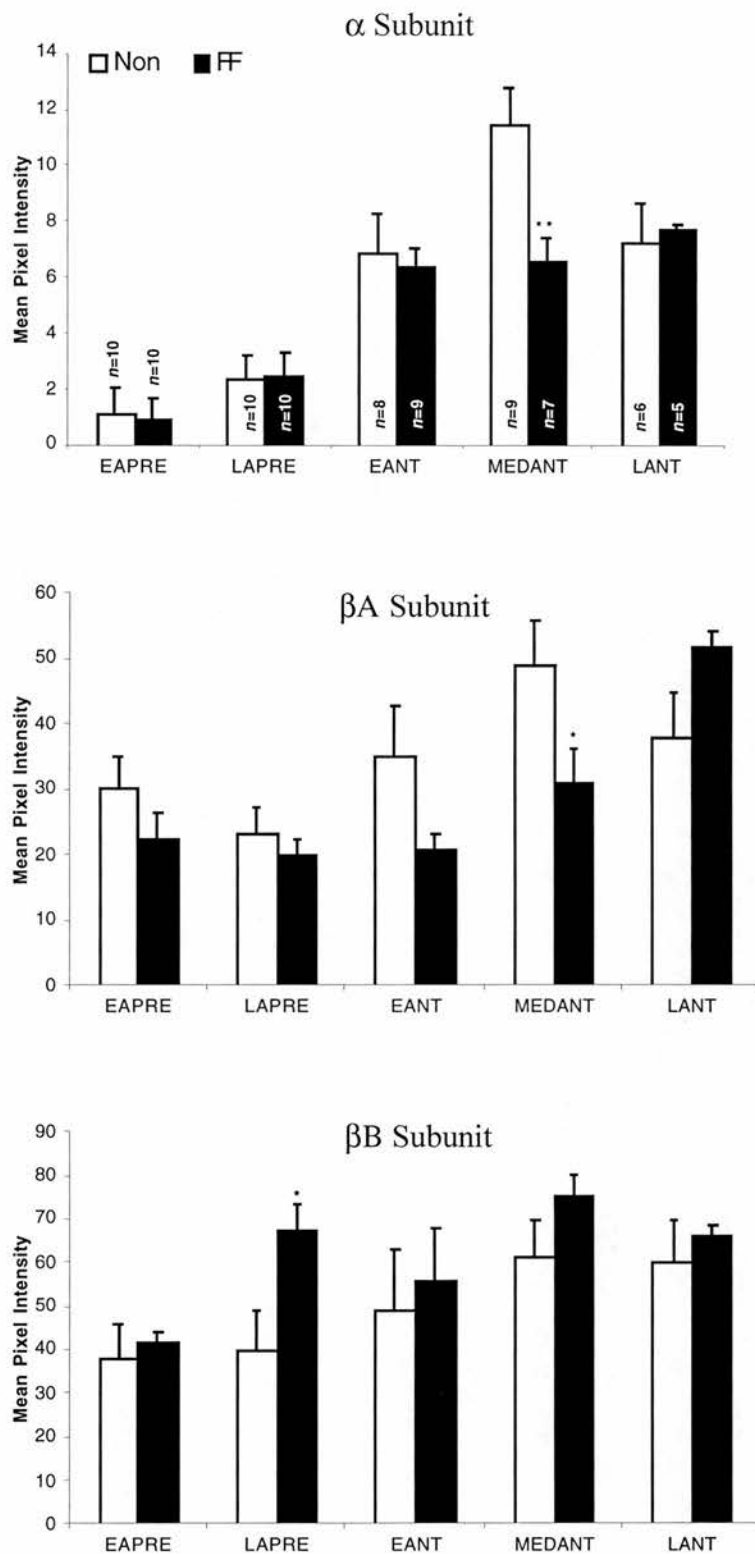


Figure 6.1 Granulosa cell expression of inhibin subunits in both carrier and non-carriers of the FecB gene. * - $P = <0.05$; ** - $P = <0.001$.

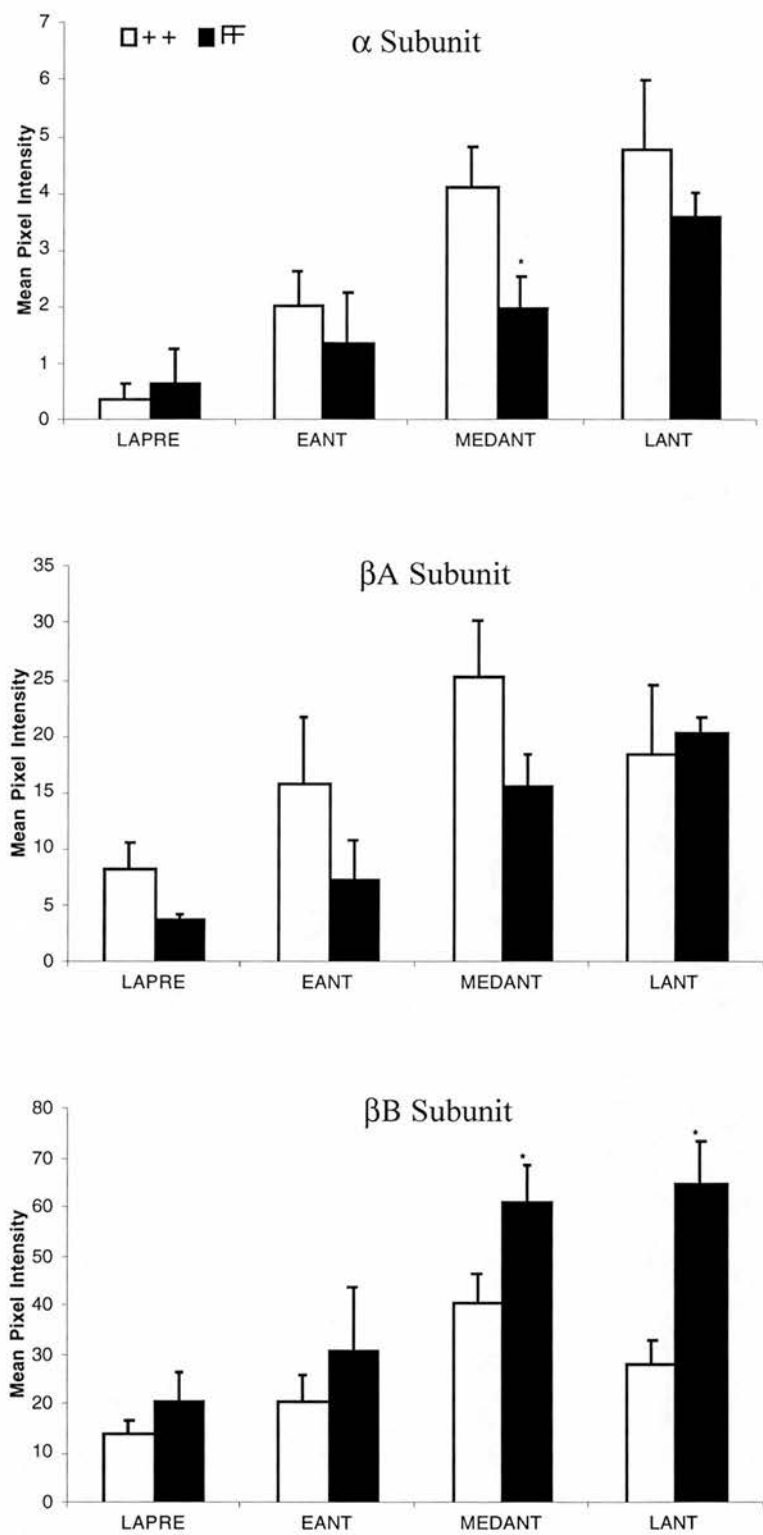


Figure 6.2 Theca cell expression of inhibin subunit protein in carriers and non-carriers of the FecB gene. * - $P < 0.05$.

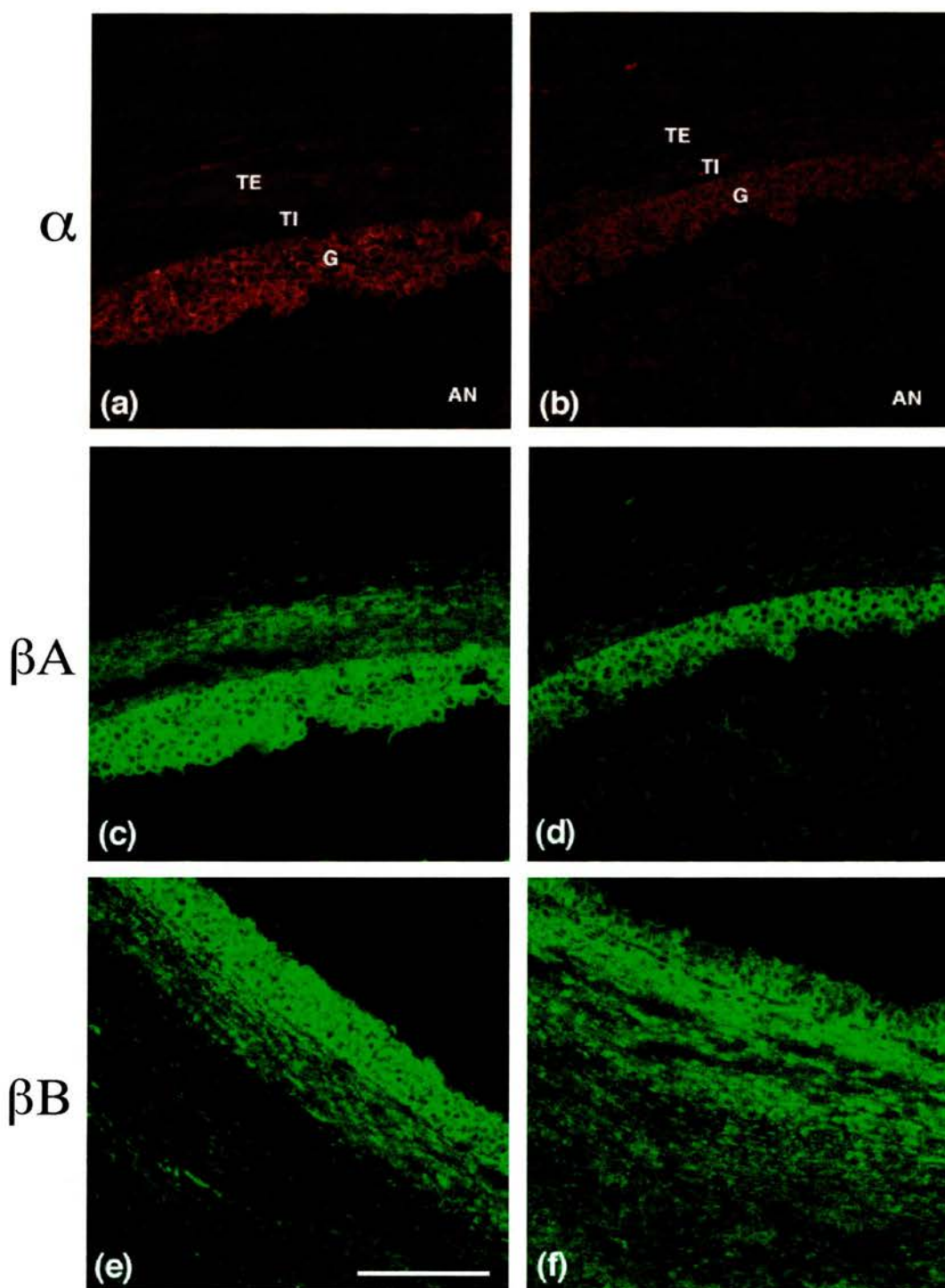


Figure 6.3 Inhibin α , β A and β B subunit protein expression in medium antral follicles in animals with (b, d, f) and without (a, c, e) the FecB gene. TE – Theca externa; TI – Theca interna; G – Granulosa; AN – Antrum. x400 magnification. Scale bar represents 100 μ m.

Chapter 7 – Activin subunit expression in ovine and murine testes

7.1 Introduction

In a number of mammalian species including the human (Groome, *et al.*, 1996) and the rat (Lanuza, *et al.*, 1999) the ovary produces both inhibin A and inhibin B, while the testis only produces the inhibin B dimer (Illingworth, *et al.*, 1996b; Woodruff, *et al.*, 1996).

The detection of inhibin subunit mRNAs and proteins in the ovaries of these species indicate the production of all three subunits in the granulosa cells. The factors which modulate the synthesis and binding of inhibin subunits to form the biopotent dimers remain unknown, however, the research presented in Chapters 4 and 5 of this thesis may suggest that expression and binding of the subunits is dependent on the stage of follicle development.

The ovine ovary expresses mRNAs and proteins for α , β A and β B inhibin subunits, as in the human and the rat; however, in the sheep only inhibin A seems to be produced with no inhibin B detectable in the circulation. FSH secretion by cultured ovine pituitary cells is considerably less suppressed by inhibin B than inhibin A (Robertson, *et al.*, 1997), suggesting that inhibin A and B may have differing biological roles.

The ram is also unique compared to males of other species studied, as although all three inhibin subunits are synthesized, inhibin A is found in the circulation with inhibin B undetectable in peripheral plasma (McNeilly, *et al.*, 2000), mirroring the situation in the ewe.

Taken together these data suggest the likely preferential dimerisation of inhibin subunits in the sheep and possibly in the gonads of other species. This hypothesis is constructed due to the fact that all three subunits are available but α subunit only binds β A subunit and not β B subunit.

The aim of this study was to examine the expression of inhibin β -subunit proteins in the testes of two species producing only one form of dimeric inhibin in order to detect any differences in the pattern of expression throughout the tissue.

Inhibin α -subunit was assumed to be present in both species as production of any inhibin form would not be possible without its presence.

7.2 Materials and Methods

Materials

7.2.1 Animal Tissue

Adult ram and rat testes sections were obtained from an in-house histological archive of fully mature animals. These sections were prepared from testes originally fixed for an appropriate length of time in 4% PFA and embedded in paraffin blocks.

7.2.2 Primary antibodies

Mouse monoclonal antibodies raised against amino acids 82-114 of the β A subunit of human inhibin (E4 clone) and amino acids 82-114 of the human β B subunit (12/13 clone) were supplied by Professor Nigel Groome, Oxford Brookes University, UK.

Methods

7.2.3 Immunohistochemistry

Sections of 5 μ m were cut from paraffin blocks containing testicular tissue; the wax was then removed from the sections using xylene and rehydrated in an ethanol gradient. The sections were incubated in a 70% methanol: 30% hydrogen peroxide solution for 30 minutes (to block any endogenous peroxidase activity) prior to a 1 hour incubation with a 20% non-specific serum in PBS blocking solution. Sections were then incubated with primary antibodies, targeted against the inhibin subunits, for one hour, washed with PBS and incubated for a further hour with the corresponding fluorescent second antibody (Sigma). The slides were mounted with 50 μ l Citifluor (Citifluor. Ltd, London) and the staining visualised using a Zeiss 510 Laser scanning confocal microscope.

7.3 Results

Immunohistochemistry was used to detect the expression of inhibin β -subunit proteins in the mature testes of two species, the sheep and the rat, in an attempt to identify any differential expression of the subunits in animals producing solely inhibin A (ram: McNeilly *et al.*, 2000) or inhibin B (male rat; Woodruff, *et al.*, 1996).

In the testis of the rat, staining for β B subunit protein was observed in the seminiferous tubules (Figure 7.1), with Sertoli cells, spermatogonium, spermatocytes and spermatids all staining positively. Only non-specific β A staining was seen in the interstitial fluid and the Leydig cells, but no β A staining was detected in the tubules. In contrast, staining for both β A and β B subunits was evident in the seminiferous tubules of the ram.

7.4 Discussion

In the testes sections examined there was a significant difference in the expression of inhibin subunit protein between the two species studied. In the ram both β A and β B subunits were detected in the seminiferous tubules, whereas the tubules of the rat were only positively stained for β B subunit. This observation is consistent with those made by other groups showing that the adult ram produces only inhibin A (McNeilly, *et al.*, 2000) and no measurable inhibin B, and that mature male rats only synthesize inhibin B in their testes (Woodruff, *et al.*, 1996).

The coupling of the β A subunit to the α -subunit of inhibin may be preferential over the binding of β B to the common α -subunit, at least in the species studied in this research. Thus in a cell-type which produces all three subunit proteins, inhibin A would be the mature form synthesised and secreted into the circulation, as detected in the peripheral plasma of the ram.

However, if the β A subunit was not expressed along with the other two proteins, the β B subunit would be free to couple to the α -subunit, producing inhibin B, as observed in the male rat.

Although this hypothesis is consistent for both sexes in the sheep, and in the male rat, female humans and rats produce both forms of inhibin and therefore express all three subunits in their ovaries. Therefore, in these animals it seems unlikely that

there is preferential binding between the β subunits and the common α subunit, and instead it is likely that the production of the two forms of inhibin is modulated by additional factors.

These factors may include the relative quantities of the subunits which are available with specific ratios required for the dimerisation of the different inhibin and activin forms. This theory is supported by previous research which suggests that at least eight times more alpha subunit than β A subunit is required for the dimerisation of inhibin A (Mason *et al.*, 1986). The fact that all three inhibin subunits are produced by the granulosa cells of the ewe and inhibin B does not seem to be synthesised, suggests the possibility that the ovine α and β B subunits can not bind to each other. One strategy which could be employed to investigate this enigma would be the transfection of a stable cell line with the genes encoding each of the ovine inhibin subunits. In this way cells could be cultured producing different combinations of the three subunits, including the synthesis of α and β B in the absence of β A. This research may indicate whether inhibin B could be produced in the ewe if β A subunit were not omnipresent in ovine follicles.

There is an additional hypothesis which may explain the preferred production of one form of inhibin over another if all three subunits are available. If the production of a form of one activin is preferred to another, for instance in a particular species activin B is formed in preference to activin A, this would reduce the level of free β B subunit for inhibin B production.

In summary, the work described in this chapter along with Western blot detection of inhibin subunits in the sheep ovary (Chapter 5), suggests that the dimerisation of inhibin subunits in the sheep may well be preferential, with β A binding to α -subunit even in the presence of the β B subunit. The results of this study are therefore in agreement with recent reports that the ewe does not produce inhibin B and that although the ram produces only inhibin A, all three subunits are present in the testes as they are in the granulosa cells of the ewe. Furthermore, this work suggests that in the male rat the production of inhibin B may only be possible in the absence of β A subunit, as in these animals the β A subunit is absent and inhibin B is produced.

To conclude, the expression of inhibin subunit proteins and the production of inhibin forms varies considerably between both species, and gender (Table 7.1). There are a number of animals, including both sexes of the sheep, which express all three of the subunits required for the production of both inhibin A and inhibin B, however, only one form of inhibin is produced. It is therefore likely that in these animals there is preferential dimerisation of the subunits in order to produce a single inhibin dimer form.

Measurements of those inhibin forms present in the plasma and subunits expressed in the gonads of additional species may well shed more light on the possible preferential order of subunit binding in the synthesis of inhibins and activins.

Species	Female			Male		
	mRNA	Protein	Inhibin Forms	mRNA	Protein	Inhibin Forms
Sheep	α , β A, β B (Chapters 3 and 4)	α , β A, β B (Chapter 4)	A (Chapters 3 and 5)	?	α , β A, β B (McNeilly, <i>et al.</i> , 2000)	A (McNeilly, <i>et al.</i> , 2000)
Rat	α , β A, β B (Findlay <i>et al.</i> , 2001)	α , β A, β B (Findlay <i>et al.</i> , 2001)	A, B (Lanuza, <i>et al.</i> , 1999)	α , β B (Klajj, <i>et al.</i> , 1990)	α , β B (Majdic, <i>et al.</i> , 1997)	B (Woodruff, <i>et al.</i> , 1996)
Human	α , β A, β B (Jaatinen <i>et al.</i> , 1994)	α , β A, β B (Eramaa, <i>et al.</i> , 1993)	A, B (Groome, <i>et al.</i> , 1996)	?	α , β A, β B (Anderson <i>et al.</i> , 1998)	B (Illingworth, <i>et al.</i> , 1996)
Pig	α , β A, β B (Li <i>et al.</i> , 1997)	?	?	α , β A, β B (Jin, <i>et al.</i> , 2001)	α , β A, β B (Jin, <i>et al.</i> , 2001)	B (Jin, <i>et al.</i> , 2001)
Cow	α , β A (Torney, <i>et al.</i> , 1989)	α , β A (Juengel, <i>et al.</i> , 1993)	A (Bleach, <i>et al.</i> , 2001)	?	?	?
Chicken	α , β A (Chen, <i>et al.</i> , 1997)	?	A (Lovell, <i>et al.</i> , 2001)	?	?	?

Table 7.1 Inhibin subunit expression and inhibin forms detected in the gonads and plasma of various species.

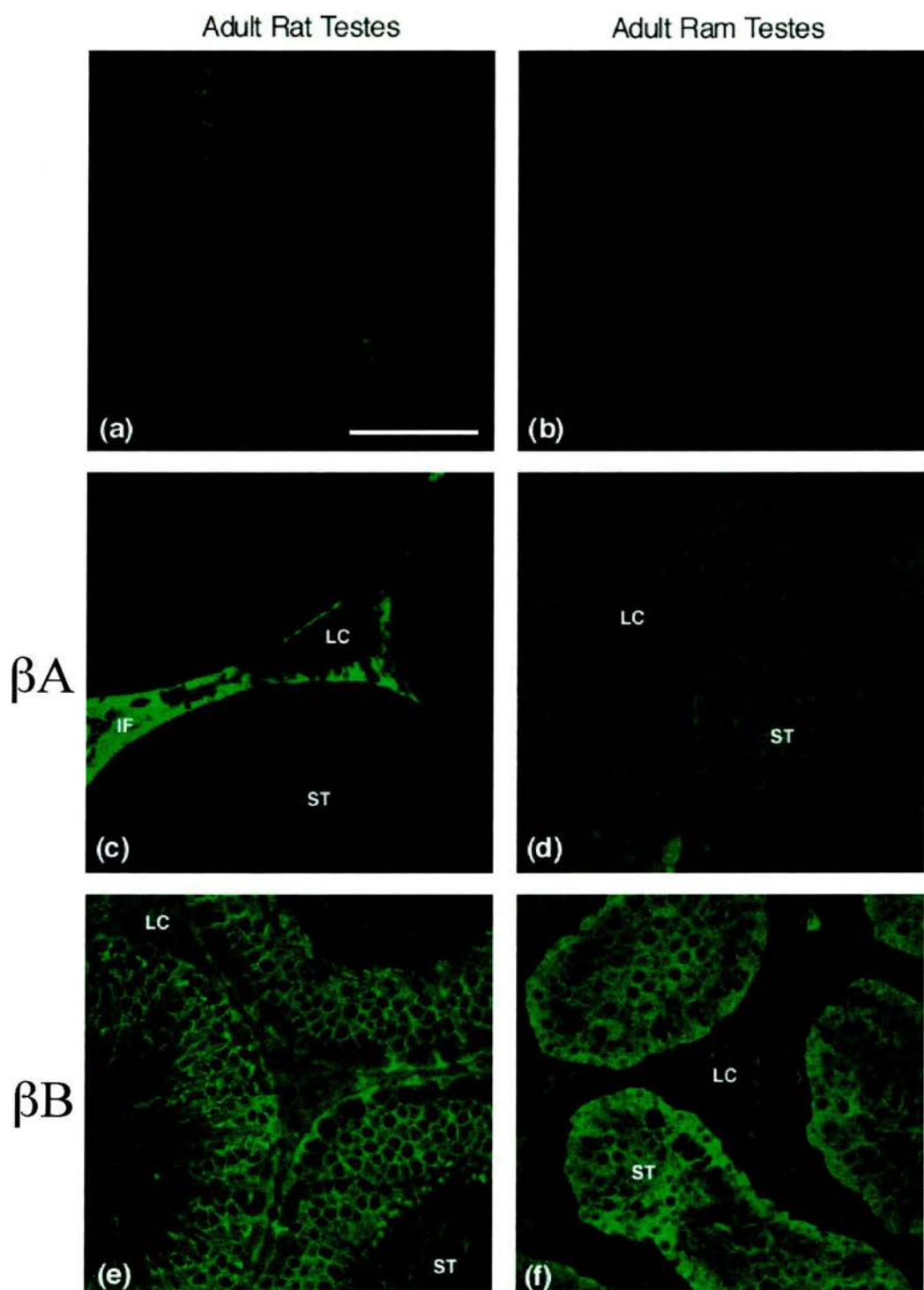


Figure 7.1 Inhibin β A-subunit (c, d) and β B-subunit staining (e, f) in the testes of the adult ram, and rat. (a) and (b) are control tissue sections. ST – Seminiferous tubule; LC – Leydig Cell; IF – Interstitial fluid. Scale bar represents 100 μ m.

Chapter 8 - General Discussion

Prior to the start of the work contained in this thesis, evidence of the involvement of inhibins and activins in the process of folliculogenesis had been widely reported. It was known that at the level of the pituitary inhibin had the ability to suppress the release of FSH (Keogh, *et al.*, 1976) with locally produced activin having the opposite effect and stimulating the secretion of the gonadotrophin from the same cells (Ling, *et al.*, 1986). A significant amount of work had also been carried out examining the local effects of inhibins and activins within the ovary with both hormones identified as modulators of steroidogenesis (Miro, *et al.*, 1991; Wrathall and Knight, 1995) and potentiators of gonadotrophic action within the ovary (Xiao, *et al.*, 1992; Findlay, 1993).

Furthermore, the development of specific two-site enzyme-linked immunosorbent assays for inhibin A and inhibin B allowed the measurement of the individual dimers in biological fluids for the first time (Groome, *et al.*, 1994; Robertson, *et al.*, 1997). However, the majority of this work was carried out in the rat and the human, with levels of the two forms of inhibin and the ovarian origin of the hormones unidentified in the sheep.

Therefore, the objectives of the work presented in this thesis were as follows:

- The identification of the source of dimeric inhibins in the ovine ovary.
- The examination of the expression of inhibin subunits throughout the ovine oestrous cycle.
- An investigation into the inhibin and activin forms produced by the ovine ovary.
- The definition of any differences seen in inhibin subunit expression in sheep with a high ovulation rate.

8.1 Follicular production of inhibin and activin in the ewe

Measurement of inhibin A in the jugular and ovarian veins confirmed that the ovary produces the majority of inhibin A detectable in the circulation of the ewe

(Chapter 3). This is in agreement with previous research identifying the ovary as the main origin of total inhibin in the sheep (Mann, *et al.*, 1989). The levels of inhibin A measured in the follicular fluids of the follicles collected for the study and in the media collected during *in vitro* incubation of these follicles, indicated that large oestrogenic follicles produce and release the greatest quantity of the hormone in relation to other follicle classes. Furthermore, it was noted that both large nonoestrogenic follicles and small antral follicles also synthesize and release a significant amount of the protein. These observations show that large oestrogenic follicles produce large quantities of inhibin A and oestradiol. These two hormones both inhibit FSH secretion by the pituitary, and therefore large oestrogenic follicles have the capability to reduce circulating levels of FSH and thus suppress the growth of subordinate follicles within the ovary. However, examination of the levels of inhibin A and FSH concentrations in peripheral blood, indicates that the fall in FSH seen in the early follicular phase is not due to an increase in circulating inhibin A. This suppression of FSH during the early follicular phase is therefore likely to be caused by the increase in oestradiol secretion from the ovary at this time. As the fall in peripheral FSH levels during the follicular phase is essential for the selection of ovulatory follicles, these observations implicate oestradiol as the primary regulator of follicle selection through the modulation of plasma FSH levels. If this is the case, suppression of FSH secretion by ovary-derived inhibin A may well be of more significance at other stages in the cycle, such as during the luteal phase when pituitary LH release, and hence ovarian oestradiol secretion, is depressed.

The fact that large follicles produce more inhibin than small follicles was supported by the immunohistochemical staining for inhibin subunits in the ovaries of the sheep (Chapter 4). The expression of α subunit was limited to the granulosa cells of those follicles classified as late preantral or antral follicles. However, as previous research has suggested that an 8-fold excess of α subunit over β A subunit is necessary for the production of inhibin A (Mason *et al.*, 1986) it is unlikely that preantral follicles produce inhibin as expression of the α subunit is minimal compared to that seen for both β subunits. In addition, the levels of β A subunit increased with follicle size with the staining most intense in large antral follicles.

Detection of inhibin A throughout the cycle revealed a number of differences between the relative contributions of the follicles size classes at the various points studied (Chapter 3). Perhaps the most significant alterations in the production and output of both inhibin A and oestradiol occurred during the early follicular phase of the cycle. Measurement of FSH in the periphery reached a nadir during this time-point reflecting the increase in negative feedback to the pituitary by oestradiol and inhibin A at this time. As expected, oestradiol secretion by large follicles reached a peak at this stage of the cycle. However, inhibin A release by large follicles was comparable with those levels detected during the mid-luteal stage of the cycle from the same follicle class. This is further evidence to support the previous hypothesis that oestradiol is the principal cause of the fall in FSH characteristic of the early follicular phase in ewes. This decline in the concentration of FSH seems to have an adverse effect on the level of inhibin A secretion by both small antral and large oestrogenic follicles, indicating that inhibin A release by these follicles is reliant upon FSH while oestradiol release from large oestrogenic follicles is more dependant on LH.

In contrast to the differences observed in the release of inhibin A throughout the ovine oestrous cycle, the expression of the subunit proteins did not vary significantly from one stage of the cycle to the next (Chapter 4). This is, in some ways, unsurprising as there was no variation seen in the level of inhibin A present in the follicular fluids of the follicles studied during the cycle, suggesting that production of the subunit proteins remains fairly constant despite changes in gonadotrophin concentrations in the blood.

The presence of inhibin β subunit in the theca cell layers of late preantral and early antral follicles is almost certainly due to the diffusion of the proteins through the cell layers from the granulosa cells and not production of the subunits by the theca cells, as mRNAs for inhibin subunits were only ever recorded in the granulosa compartment of follicles. This ability of β subunits, as activin dimers, has been noted previously, with the molecule able to travel up to 7 cell lengths through embryonic *Xenopus* tissue, via rapid diffusion and the establishment of an activin gradient across the tissue. The fact that β subunit protein was detected in small preantral follicles, with undetectable mRNA for the subunits, has been reported previously (McNatty,

et al., 2000) as well as being observed in this work. We propose that the most likely source of β subunit staining in small preantral follicles is activin, which has diffused from the granulosa cells of larger antral follicles.

Research has shown that activin stimulates the growth of preantral mouse follicles from immature animals, while maintaining an inhibitory effect on preantral follicles in adult mice (Yokota, *et al.*, 1997). Furthermore, additional studies have provided evidence that activin secreted by secondary follicles causes preantral mouse follicles from adult mice, cultured *in vitro*, to remain quiescent in the resting stage of growth (Mizunuma, *et al.*, 1999).

Therefore, if the β B subunit staining seen in the theca cells represents activin, it is possible that it is able to diffuse through the theca cell layers of large follicles, and for some distance through the stromal tissue of the ovary, to exert a likely negative effect on the growth of neighbouring preantral follicles. In addition, the intense staining for β B subunit proteins seen in the *zona pellucida* may implicate activin B in signalling between the cumulus granulosa cells and the oocyte.

It is possible that diffusion of activin from antral follicles may play a significant role in the precocious development of ovulatory follicles in *Fec^B* ewes. As documented in Chapter 6 of this thesis, the diffusion of β B subunit protein through the theca cell layers is significantly greater from larger antral follicles in animals carrying the *Fec^B* gene. Although activin has been shown to exert a negative effect on preantral follicles in mice, the dimer has the ability to enhance the effect of FSH on the maturation of granulosa cells within the ovary (Xiao, *et al.*, 1992). However, as recent research has suggested that the Booroola phenotype is caused by an alteration in BMP signalling pathways (Mulsant, *et al.*, 2001; Souza, *et al.*, 2001), it is likely that alterations in inhibin subunit expression are a result of this disruption to a pathway involved in the induction of cellular differentiation.

8.2 Molecular forms of inhibin in the sheep

The work presented in Chapters 3 and 4 of this thesis employed antibodies targeted against regions of the mature α , β A and β B inhibin subunits. However, as well as identifying proteins containing the mature subunit forms, these antibodies will also bind to precursor forms which are also present in the ovaries of the ewe.

The fact that the immunoassay utilised in Chapter 3 uses a sandwich technique with two antibodies used to specifically detect mature inhibin dimers, allowed the detection of dimerised inhibin A. However, to investigate the forms of inhibin subunits identified with immunostaining in Chapter 4, and also to detect possible inhibin B dimers, a Western blotting protocol was employed.

The absence of inhibin B dimers in the follicular fluid of ovine follicles is in agreement with recent research which, using a validated specific assay, was unable to detect inhibin B in the blood of the male or female sheep (McNeilly, 2000).

Thus, although the granulosa cells of antral follicles may synthesize inhibin B as well as inhibin A, it is unlikely that it is secreted into the bloodstream or follicular fluid.

Inhibin B has been identified as the major inhibin form in the human male and the male rat, and has also been shown to play a significant role in folliculogenesis in the females of these species, which both produce inhibin A in addition to inhibin B. The fact that inhibin B is not present in the plasma of the ewe or ram prompts the question, why is inhibin B not required in the sheep, if it plays a significant role in other species?

Inhibin B has less activity than inhibin A in suppressing FSH secretion by ovine pituitary cells (Robertson, *et al.*, 1997); therefore the primary role of inhibin B in the sheep may be as a paracrine/autocrine factor within the ovary rather than as a 'true' hormone.

Further research centred around the role of inhibin B in those species in which it is found may offer an explanation for its absence in the sheep.

Furthermore, the detection of α , β A and β B subunits in the same gonadal cells in both male and female sheep, would suggest that the production of both inhibin A and B should occur as observed in the female human and rat (Groome, *et al.*, 1996; Lanuza, *et al.*, 1999).

As all three subunit proteins are present in the ovine ovary and testis, why is inhibin B not secreted in either sex? One possible explanation is that inhibin A is produced preferentially in these animals.

There are a number of possible theories as to why this selective production of inhibin A might occur in the sheep. The binding of α subunit to the β B subunit may not be physically possible due to slight differences in the amino acid sequences of the two subunits, which may prevent the formation of inhibin but do not interrupt the dimerisation of the subunits to form activin. It is also possible that the affinity of β B subunits for each other is stronger in the sheep than their affinity for α subunits, so that as β B subunits are produced, activin is formed, leaving little β B subunit free in the granulosa to produce inhibin. It is also important to note that in the rat testis there is no inhibin A produced due to a lack of β A subunit expression, and in the rat ovary inhibin A is only produced when β A subunit is expressed late in folliculogenesis. These observations provide additional evidence for the hypothesis that preferential dimerisation of inhibin subunits exists in these species.

8.3 General conclusions

The work described in this thesis shows that large oestrogenic follicles are the major source of inhibin A in the ewe, with both small antral and large non-oestrogenic follicles contributing significant amounts. Furthermore, the secretion of inhibin A by small antral and large oestrogenic follicles falls during the early follicular phase, concomitant with a decline in peripheral FSH. Oestradiol secretion by large oestrogenic follicles remains unaffected by the fall in FSH demonstrating the dependence of inhibin A release on FSH, while oestradiol secretion is able to transfer its stimulus from FSH to LH.

In addition, we suggest that inhibin β subunits, possibly in the form of activin, are able to diffuse through the ovine ovary, exerting an effect on the growth and differentiation of neighbouring follicles. This diffusion is enhanced in the ovaries of ewes with an increased ovulation rate and may offer an explanation for the early maturation of ovulatory follicles in these animals.

Finally, in contrast to females from other species, we have shown that it is unlikely that the ewe secretes inhibin B into the circulation, and that the binding of subunits during the formation of inhibins and activins in the sheep and a number of other species may well be preferential.

These conclusions are summarised in Figure 8.1, which shows the interactions between inhibin and activin produced by certain ovarian follicles and their effects on FSH secretion (endocrine) and action (autocrine/paracrine), and their hypothesised effects on adjacent follicles within the ovary.

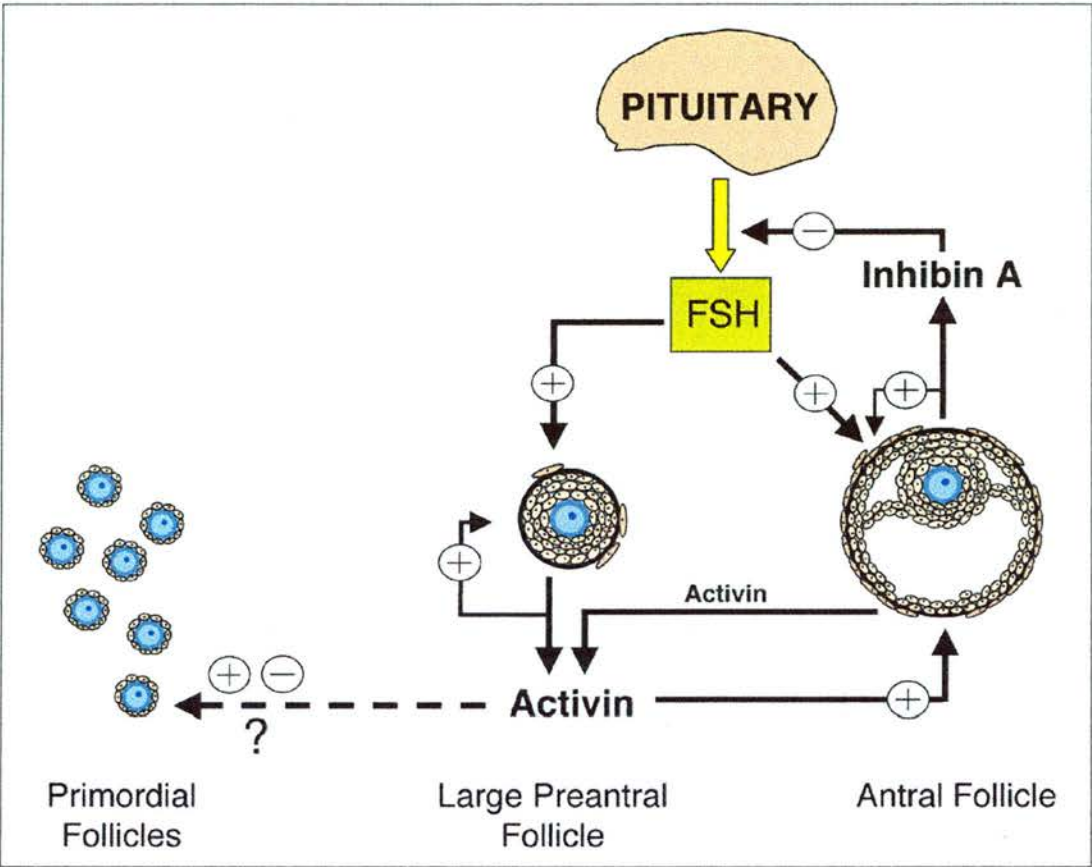


Figure 8.1 Summary diagram showing the involvement of inhibins and activins in the process of follicle development in the sheep.

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Appendix

Assay Protocols

Oestradiol Assay

Day 1

	Standard/QC	Buffer	Sample	Medium	AB
Total Counts	-	-	-	-	-
NSBs	-	200µl	-	50µl	-
B0s	-	100µl	-	50µl	100µl
Standards	100µl	-	-	50µl	100µl
QCs	50µl	100µl	-	-	100µl
Samples	-	100µl	50µl	-	100µl

Set up tubes as above, the primary antibody used (AB) is a sheep anti-oestradiol antibody (BW 26/9/82) diluted in PGel (assay buffer) to relevant tubes (working dilution is 1:500,000).

Add 100ul of the ^{125}I E₂ tracer (10,000-12,000cpm/100µl) diluted in PGel. Leave the tubes overnight at 4°C.

Day 2

Add second antibody, 125µl SAGS (sheep anti-goat serum) diluted 1:20 in PGel and 125µl normal sheep serum (1:200 in PGel). Leave the tubes overnight at 4°C.

Day 3

Add 250ul of PGel containing 1% Tween 20 to all tubes except TCs and NSBs, spin at 3000rpm for 30 minutes at 4°C. Drain supernatant and count pellets on gamma counter for 60 seconds.

Androstenedione Assay

Day 1

	Standard/QC	Buffer	Sample	Medium	AB
Total Counts	-	-	-	-	-
NSBs	-	200µl	-	50µl	-
B0s	-	100µl	-	50µl	100µl
Standards	100µl	-	-	50µl	100µl
QCs	50µl	100µl	-	-	100µl
Samples	-	100µl	50µl	-	100µl

Set up tubes as above, primary antibody (AB) used is anti-androstenedione RB3 at a final working dilution of 1:3,000,000.

Add 100ul of the ^{125}I A₄ tracer (10,000-12,000cpm/100µl) diluted in PGel. Leave the tubes overnight at 4°C.

Day 2

Add second antibody, 125µl SAGS (sheep anti-goat serum) diluted 1:20 in PGel and 125µl normal sheep serum (1:200 in PGel). Leave the tubes overnight at 4°C.

Day 3

Add 250ul of PGel containing 1% Tween 20 to all tubes except TCs and NSBs, spin at 3000rpm for 30 minutes at 4°C. Drain supernatant and count pellets on gamma counter for 60 seconds.

Progesterone Assay

Day 1

	standards/QC	Buffer	Sample	Ovx plasma	AB
total counts	-	-	-	-	-
NSB's	-	200 µl	-	50 µl	-
BO's	-	100 µl	-	50 µl	100 µl
standards	100 µl	-	-	50 µl	100 µl
QC's	50 µl	100 µl	-	-	100 µl
sample	-	100 µl	50 µl	-	100 µl

Set up tubes as above. Primary antibody (AB) used is lyophilized antiserum (SAPU S235-201) with a working dilution of 1:30,000.

Add 100 µl of ^{125}I P4 tracer (approximately 15,000 cpm), diluted in Phosphate Citrate Buffer (PCB) with 10 mg/10 ml ANSA (8-Anilino-1 Naphthaline Sulphonic Acid) and without gelatin, to all tubes. Mix tubes and incubate for at least three hours at room temperature.

Add 100µl DARS and 100µl NRS to all tubes (except total counts), mix the tubes and leave overnight at 4°C.

Day 2

Add 1ml 0.9% saline to all tubes (except total counts), spin 3000 rpm for 30 minutes at 4°C. Discard the supernatant and count remaining pellets on gamma counter for 60 seconds.

Buffers

PCB

Na_2HPO_4	17.85 g	35.70 g
Citric acid powder	7.75 g	15.5 g
Thiomersalate	0.1 g	0.2 g
Distilled water	1 L	2 L
pH to 6.0		
Gelatin (not in tracer buffer)	1.0g	

FSH Assay

Day 1

	standards/QC	Buffer	Sample	AB
Total counts	-	-	-	-
NSB's	-	350 μ l	-	-
BO's	-	300 μ l	-	50 μ l
Standards	150 μ l	-	-	50 μ l
QC's	150 μ l	150 μ l	-	50 μ l
Sample	-	150 μ l	150 μ l	50 μ l

Set up tubes as described above, the primary antibody used is NIDDK-NIH anti-ovine FSH-1 used at a working dilution of 1:12000.

Day 2

Add 50 μ l 125 I labeled NIAMMD oFSH-19 to all tubes in the assay (approximately 15,000 cpm/tube), mix and incubate at 4oC overnight.

Day 3

Add 100 μ l donkey anti-rabbit serum (DARS; SAPU), diluted 1:32 in assay buffer, and 100 ml normal rabbit serum (NRS; SAPU), diluted 1:400 in assay buffer to all tubes (except the total counts). Mix the tubes and incubate overnight at 4oC.

Day 4

Add 1ml 0.9% Saline, 4% PEG, 0.2% Triton X to all tubes except the total counts. Spin for 25 minutes at 3000rpm, 4oC. Decant the supernatant, allow tubes to dry and count pellets using a gamma counter for 60 seconds.

Inhibin A Assay

Day 1

Add 100µl of standards/QCs/samples/blanks to an eppendorf tube. Add 50µl of 6% SDS solution. Boil tubes at 100°C for 3 minutes. Cool and add 50µl of freshly prepared 6% hydrogen peroxide followed by 100µl of assay buffer. Incubate at room temperature for 30 minutes.

E4 coated plates? Add 80µl of sample/standard/QC per well to an E4 precoated plate in duplicate. Incubate at room temperature overnight. Plates should be covered with a plate sealer and placed in a sealed box with damp paper towels.

Day 2

Discard plate contents and wash x20 with wash buffer. Make a 1/1000 dilution of PPG biotinylated antibody in assay buffer containing 1% (v/v) mouse serum and add 50µl to each well. Incubate at room temperature for 3 hours. Wash x20 with wash buffer. Make a 1/1000 dilution of streptavidin alkaline phosphatase in assay buffer containing and add 50µl to each well. Incubate at room temperature for 1 hour. Wash x20 with wash buffer. Add 50µl of alkaline phosphatase substrate per well. Incubate at room temperature for 2 hours. Add 50µl of amplifier. Wait until the OD of the top standard is between 1-1.5 then stop with 50µl of 0.4M HCl. Read at 490nm on a plate reader.

Assay Buffers

Oestradiol/Androstenedione Assay Buffer

0.05M PBS with 0.1% Gelatine and 0.1% sodium azide (PGel)

	g/2litre
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	4.6
$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	12.6
NaCl	18.0
Sodium azide	2.0
Gelatine	2.0
pH 7.0-7.2	

Progesterone Assay Buffer

0.5 M Stock Phosphate Buffer (1L)

	g/litre
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	19.5
$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	66.75
Distilled water	1 litre

Phosphate Assay Buffer (2L)

	g/2Litre
NaCl	18.0
Gelatine	2.0
Sodium Azide	0.2
0.5M Stock phosphate buffer	200 ml
Distilled water	1800 ml

FSH Assay Buffer

0.5 M Stock Phosphate Buffer (1L)

	g/litre
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	19.5
$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	66.75
Distilled water	1 litre

0.075M PBS (5L)

	g/5 litre
NaCl	43.85
Thiomersal	0.5
0.5M Phosphate Buffer	750 ml
Distilled water	4250 ml

1% BSA/PBS (Store at 4oC)

	g/litre
Bovine serum albumin	10
0.075M PBS	1000 ml

Inhibin A Buffers

Amido black solution (0.06% w/v)

Dissolve 60mg of amido black in 100ml of deionised water.

Assay (diluent buffer- blue coloured)

Tris	1.21g
NaCl	0.9g
BSA	10g
Triton X-100	5ml

Dissolve in 80ml deionised water and adjust pH to 7.5 with HCl. Add 0.1g of sodium azide and 2ml of 0.06% amido black. Make up to 100ml with further deionised water.

Hydrogen peroxide

Hydrogen peroxide (30%)	1ml
Deionised water	4ml

Wash buffer

Tris	75.5g
NaCl	112.5g
Tween 20	6.3ml

Dissolve in about 300ml deionised water. Adjust pH to 7.5 with HCl then make up the volume to 500ml with further deionised water. Dilute 1:25 with deionised water before use

Immunohistochemistry

Double Fluorescent Detection

Protocol

Day 1

- Dewax sections in xylene for 5 mins.
- Rehydrate sections using alcohol gradient.
- Boil in citrate buffer for 5 minutes.
- Wash slides 2 x 5 minutes in PBS.
- Block in blocking solution for 30 minutes.
- Make up primary antibody/antibodies in blocking solution. For double fluorescent staining the antibodies must be from different species.
- Add antibody to slides (approx 50µl/slide).
- Incubate overnight at 4°C

Day 2

- Wash slides 2 x 5mins in PBS
- Incubate slides with second antibodies raised against the primary antibody species (one FITC, one TRITC conjugate), diluted to 1:50 in blocking solution, for 1 hour.
- Wash 2 x 5 minutes in PBS.
- Add 50µl Citifluor (Glycerol/PBS solution) to each slide.
- Add a coverslip and use mounting adhesive to seal the edges.
- Visualise on a confocal microscope.

Buffers

Phosphate buffered saline (PBS)

Sigma Tablets 1 per 200mls dH₂O

Blocking Solution

16mls PBS, 4mls normal porcine serum and 1g BSA

Western Blotting

Protocol

- Sample homogenised (tissue) in 1 ml protein extraction buffer.
- Diluted to 1:10 in extraction buffer
- Protein concentration measured x3 using Genequant
- Dilution of original extract calculated to give 2µg/µl

- 20µl of sample mixed with 5µl of sample application buffer and loaded onto 4-20% Tris-Glycine precast gel
- Gel run at 120V, 26ma for 2 hours
- Gel removed from cassette and placed into sandwich with PVDF membrane and whatman paper
- Transferred in blotting module at 30V, 400ma for 2 hours

- Membrane blocked with Porcine serum (5% in TBS-T) for 1 hour
- Washed 3x 10 minutes with TBS-T
- Incubated at room temperature with primary antibody (1:1000) with 5% porcine serum in TBS-T for one hour
- Washed x3 TBS-T
- Incubated at room temperature with alkaline phosphatase linked secondary antibody (1:20000) with 5% porcine serum
- Washed x3 TBS-T
- Membrane laid flat in square petri dish, 2ml ECF substrate added evenly to surface left in dark at room temperature for 20 minutes
- Gel stained Coomassie solution for five minutes and destained for ~30 minutes
- Membrane lifted and rested on whatman paper and dried in dark at room temperature for 20 minutes
- Read on Storm Phosphoimager at 800V

Solutions

Protein Extraction Buffer

20mM Tris HCl

150mM NaCl

Protease Inhibitor (1 tablet – Boehringer Mannheim)

TBS-T (1L)

50 ml 1M Tris HCl

50 ml 3M NaCl

0.5ml Tween

900ml dH₂O

Tank Buffer

25mM Tris HCl

0.2M Glycine

0.1% SDS

Transfer Buffer

192mM Glycine

25mM Tris Base

20% Methanol

0.1% SDS

Sample Application Buffer (5X – Reducing)

2.5mls Glycerol

0.185g Tris

1.25mls 20% SDS

15.6mg Bromophenol blue

0.625mls β-mercaptoethanol

0.32mls dH₂O

Sample Application Buffer (5X – Non-Reducing)

2.5mls Glycerol

0.185g Tris

1.25mls 20% SDS

15.6mg Bromophenol blue

0.95mls dH₂O

Gel Staining Solution

0.5% w/v Coomassie Brilliant Blue

30% Methanol

10% Glacial Acetic Acid

60% dH₂O

Gel Destaining Solution

30% Methanol

10% Glacial Acetic Acid

60% dH₂O

In situ Hybridisation

Probe Labelling

<u>Reaction Mix</u>	<u>Full Reaction</u>	<u>Half Reaction</u>
5X Transcription Buffer	4µl	2µl
100mM DTT	2µl	1µl
rRNasin	20 units (1µl)	0.5µl
rNTPs	4µl	2µl
Linearised Plasmid DNA	1µl	0.5µl
³⁵ S UTP	100µCi	50µCi
Polymerase (T3 or T7)	1µl	0.5µl
DEPC H ₂ O to	20µl	10µl

Incubate the above mixture for 1 hour at 37oC then add the following:

	<u>Full Reaction</u>	<u>Half Reaction</u>
DEPC H ₂ O	10µl	20µl
tRNA (10mg/ml)	10µl	10µl
5X Transcription Buffer	8µl	8µl
rRNasin	1µl	1µl
<u>RQ1 RNase-free Dnase</u>	<u>1µl</u>	<u>1µl</u>
Final Volume	50µl	50µl

Calculate Specific activity and % incorporation for each probe as described in Chapter 2.

Pre-hybridisation

- | | |
|---|-------------|
| 1) Dewax in histoclear | 2 x 10 mins |
| 2) 100% Ethanol | 2 x 2 mins |
| 3) 95 – 80 – 60 – 30% Ethanol | 2 mins each |
| 4) 0.85% NaCl | 5 mins |
| 5) 1X Phosphate Buffered Saline (PBS) | 5 mins |
| 6) 4% Paraformaldehyde (PFA) in PBS | 20 mins |
| 7) 1X PBS | 2 x 5 mins |
| 8) Proteinase K (1mg/ml) | 7.5 mins |
| 400mls: 40 ml 0.1M EDTA | |
| 40 ml 1M Tris | |
| 320 ml DEPC | |
| 800ml Proteinase K | |
| 9) 1X PBS | 5 mins |
| 10) 4% PFA in PBS | 5 mins |
| 11) DEPC H ₂ O | 10 secs |
| 12) 2X SSC | 2 mins |
| 13) 0.1M Triethanolamine-HCL and Acetic anhydride | 10 mins |
| 14) 2X SSC | 2 mins |
| 15) 60 – 80 – 95 – 100% Ethanol | 2 mins each |
| 16) Chloroform | 5 mins |
| 17) 100% Ethanol | 2 mins |
| 18) 95% Ethanol | 2 mins |
| 19) Dry slides in fumehood. | |

Hybridisation Buffer

Dextran sulphate	2g
DEPC H ₂ O	7ml
Deionised formamide	10ml
20X SSC	4ml
Denharts (100X)	200µl
Yeast tRNA (100mg/ml)	100µl
1M DTT	1ml

Post-Hybridisation

- 1) Remove parafilm
- 2) Wash slides thoroughly in 2X SSC at 55°C.
- 3) RNaseA (20mg/ml) in 2X SSC at 37°C 60 mins
- 4) 2X SSC, 0.1% β-mercaptoethanol 15 mins
- 5) 1X SSC, 0.1% β-mercaptoethanol 15 mins
- 6) 1X SSC, 0.1% β-mercaptoethanol, 50% formamide 15 mins
- 7) 0.1X SSC, 0.1% β-mercaptoethanol 2 x 15 mins
- 8) 60 – 80 – 95 – 100% Ethanol 2 mins each
- 9) Dry slides in fumehood.
- 10) Expose, develop and fix as described in Chapter 2.